



**ANTIBACTERIAL ACTIVITY OF *PSEUDOMONAS* SP. ISOLATED FROM
RHIZOSPHERE CITRUS SOILS AGAINST *STAPHYLOCOCCUS* SPECIES AND
OTHER PATHOGENS**

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ABSTRACT

The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large active groups of bacteria. *Pseudomonas fluorescens* is an antibiotic producer and has a wide spectrum of antimicrobial activity against *Salmonella typhimurium*, *Bacillus subtilis*, *Proteus vulgaris* and *Candida albicans*. The aim of this study was to investigate the antibacterial activity of a compound from *Pseudomonas* sp. against methicillin-resistant *Staphylococcus aureus* (MRSA) strains and other pathogenic bacteria. Samples were serially diluted and 0.1 mL of sample was spread on King's B agar plates. The isolated bacteria was identified by morphological and biochemical tests. The results were confirmed using molecular methods. Screening of antibacterial activity of the isolated *Pseudomonas* sp. from rhizosphere soil was performed by agar-well diffusion assay. After precipitation by aluminum sulfate and dialysis, the isolates were analyzed by SDS- PAGE analysis. Finally, phylogenetic studies were performed using 16S rRNA sequences. The isolated strain was a gram negative bacterium, aerobic and from *Pseudomonas* genus. The cell-free culture supernatants and purified proteins obtained from the isolated strain shown a significant antimicrobial activity against MRSA, other *Staphylococcus aureus* strains and *Escherichia coli*, on Muller-Hinton agar. Moreover, the size of the antibacterial agent on SDS-PAGE was approximately 50 kDa. The profound zone of

inhibition, has warranted a strong antibacterial agent, especially for gram positives bacteria such as MRSA and other *S. aureus* strains.

Keywords: Rhizosphere - MRSA, Antimicrobial agent - *Pseudomonas* sp. - Phylogenetic analysis

INTRODUCTION

The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large active groups of bacteria [1]. It could be considered as a natural habitat for screening studies to find a suitable source for novel antibiotic-producing microorganisms. Several rhizobacteria have been used extensively as biological agents to control many soil borne plant pathogens [2, 3]. Rhizobacteria exert their beneficial effect on plants by various mechanisms including siderophores, hydrogen cyanide (HCN) production, antibiotics, lytic enzymes, competition or by inducing systemic resistance [4, 5]. Over the last few decades, the number and proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in different countries has increased due to the rise of epidemics in humans [6, 7] and animals, such as dogs, cats, cattle, pigs and exotic species [8, 9]. In a study comparing two neonatal ICUs, the cost of instituting control measures in a stepwise, delayed approach was US\$ 49–69 million, while the cost of introducing effective and immediate measures was US\$ 1.3 million

[10]. Another study calculated that the total cost per case of bacteremia that was caused by an antibiotic-resistant strain, including MRSA (50% of the cases), was US\$ 88,445 [11].

Recently, some natural antibacterial agents, such *Quercus dilatata*, *Hylomecon hylomeconoides*, *Eleutherine Americana*, *Chelidonium majus* Linn. (Papaveraceae) and *Tabebuia avellanedae* compounds, have been tested against MRSA [12, 13]. The ability of antibacterial compounds obtained from other bacteria to inhibit methicillin-sensitive *S. aureus* (MSSA) and MRSA has also been tested [14, 15]. Different *Pseudomonas* species, are strong antibiotic producers [16] and have a wide spectrum of antimicrobial activity against *Salmonella typhimurium* [17] *Bacillus subtilis*, *Proteus vulgaris* and *Candida albicans* [18]. These characteristics make *Pseudomonas* species good candidates for utilization as seed inoculants, root dips for biological control of soil-borne plant pathogen and also as antibacterial agents. It also has got broad spectrum activity against *S.*

aureus, *E. coli* and *Aeromonas hydrophila* [19, 20].

The aim of this study was to investigate the antibacterial activity of the obtained compounds from a naturally isolated *Pseudomonas* sp. strain against MRSA strains and other pathogenic bacteria. Besides, its phylogenetic analysis was performed to prepare better understanding about its homology with other related strains.

MATERIALS AND METHODS

Isolation of *Pseudomonas* sp.

The rhizosphere soil sample (1 g) was suspended in 99 mL of sterile saline solution. Samples were serially diluted (10⁻⁵) and 0.1 mL of sample was spread on King's B agar plates and incubated at 37°C for 24 h. The isolated bacteria was confirmed by morphological and biochemical tests, based on Bergey's manual of systematic bacteriology [21].

Evaluation of the antibacterial effects

Screening of antibacterial activity of *Pseudomonas* sp. isolated from rhizosphere soil by agar-well diffusion assay. Antibacterial activity of *Pseudomonas* sp. isolated from rhizosphere soil was tested against target bacterial pathogens of health significance like *S. aureus* and MRSA by in vitro techniques using Muller-Hinton agar plates at 37 °C for 24 h. MRSA and *S. aureus*

suspensions of 10⁸ colony forming units (CFU)/mL were grown to log phase, and the well diffusion was treated with the antibacterial compounds. The plates were incubated at 37°C for 24 h, and the size of the inhibition halos diameter was evaluated (mm). The antibacterial effect was determined by measuring the size of inhibited halos formed around the wells.

16S rRNA gene sequencing

The chromosomal DNA genome used for polymerase chain reaction (PCR). It was prepared using phenol-chloroform method [22]. The DNA fragments containing 16S rRNA were amplified from the chromosomal DNA with primers pairs 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3) [23]. PCR reactions were performed in a thermal cycler (Bio-Rad Laboratories, Hercules, USA) in a total volume of 50 µL containing Master Mix (Takara, Japan). Amplification consisted of denaturation step at 94°C (1 min), annealing step at 59°C (1 min) and extension step at 72°C (1 min). The first cycle was preceded by incubation for 5 min at 94°C. After 35 cycles, there was a final 10 min extension at 72°C. Negative controls containing no DNA template were included in parallel. PCR products were separated in a 1.5% (w/v) agarose gel and were

subsequently visualized by ultraviolet (UV) illumination after ethidium bromide staining.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To estimate the molecular mass of bioactive peptides, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% acrylamide gels. A low molecular mass protein marker with size ranging from 15 to 200 kDa (Sigma-Aldrich, Germany) was used as standard. To determine the apparent molecular mass of the bacteriocin, the gel was cut into two vertical parts after SDS-PAGE. The part of the gel containing the molecular mass marker and the samples was stained with Coomassie blue R-250 for 24 h, while the remaining part, containing only samples, was extensively washed with regularly replaced sterile MilliQ water for Repeat 3 times and decolorizing for 3 hours.

Bioinformatic analysis of the amplified sequence

Sequence similarity searches were done on the NCBI databases with BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The MultAlin software, version 5.4.1 was used which creates a multiple sequence alignment from a group of related sequences using progressive pairwise alignments [24]. The MEGA software, version 6, an integrated

bioinformatic tool for inferring the phylogenetic trees and estimation of the divergence times [25] was used to conduct the phylogenetic studies and compare the retrieved sequences. The conserved domains among these sequences were also identified. The CLC sequence viewer software (Qiagen, Aarhus, Denmark) version 7.5, a bioinformatic tool for annotating multiple sequence alignments, shading and alignment editing, was used to compare the retrieved sequences. The conserved domains among these sequences were also identified.

RESULTS AND DISCUSSION

Characteristics of the isolated strain

The isolated strain was found to be an aerobic, Gram-negative rod shaped bacterium. Its biochemical characteristics were listed in Table (1).

Based on morphological, biochemical and molecular methods using 16S rRNA gene analysis, the identified strain was identified as belonging to the Pseudomonaceae family. Its DNA sequence was recorded in the NCBI under the accession number KM487190.1 as *Pseudomonas* sp. Besides, the strain was phylogenetically characterized and identified using the closest relative strains (Figure 1). The evolution history was inferred by using the Maximum Likelihood method. The bootstrap consensus tree inferred from 500

replicates It showed 97-100% homology to the 16S small subunit rRNA of other *Pseudomonas* species. The analysis involved 18 nucleotide sequences (accession numbers in parenthesis).

The multiple sequence alignments, the similarity between the studied sequences and the conserved domains among the studied sequences were shown with a color scale (Fig. 2). The blue residues are the least conserved and white residues are the most conserved. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The consensus sequence is also shown in the below of seven studied sequences. Besides a color plot (green for the least conserved and white for the maximum ones) shows the conservation extent of each domain.

Antimicrobial activity assay

The antimicrobial activity of cell-free culture supernatant (CFCS) and purified proteins (PP) obtained from the isolated *Pseudomonas* strain were tested against MRSA and

Staphylococcus aureus, *E.coli* and O157:H7 pathogenic bacteria by well diffusion assay. The results are shown in Table II and Figure 3. The CFCS and PP exhibited some significant antibacterial effects on a broad range of bacterial species.

Purification and analysis of the antibacterial agents

Pseudomonas sp. was grown on BTM broths. Antibacterial activity was observed in supernatants of cell-free culture incubated at 37°C. Antibacterial from CFCS was recovered by ammonium sulfate precipitation. In this step, some antibacterial agents can precipitate at different ammonium sulfate concentrations. Salted-out proteins were precipitated by dialysis. Analysis of the fractions isolated after dialysis by SDS-PAGE, and direct detection of antimicrobial activity on the electrophoresis gel indicated that molecular mass of the spot is approximately between 50-52 kDa (Fig. 4).

Table I: Morphological and biochemical characterization of *Pseudomonas* sp. isolated from rhizosphere soils

Characteristic	<i>Pseudomonas</i> sp. strain
Colony morphology	Medium, Smooth
Gram staining	Short Rod, Gram negative
Motility	+
Catalase	+
Oxidase	+
Potassium Nitrate	-

L-Tryptophan	-
D-Glucose (Fermentation)	-
L-Arginine	+
Urea	-
Esculin	-
Ferric citrate	-
Gelatin	+
Nitrophenyl- β -D-galactopyranoside	-
D-glucose (Assimilation)	+
L-arabinose	-
D-mannose	-
D-mannitol	+
N-acetyl-glucosamine	-
D-maltose	-
Potassium gluconate	+
Capric acid	+
Adipic acid	+
Malic acid	+
Trisodium citrate	+
Phenylacetic acid	-

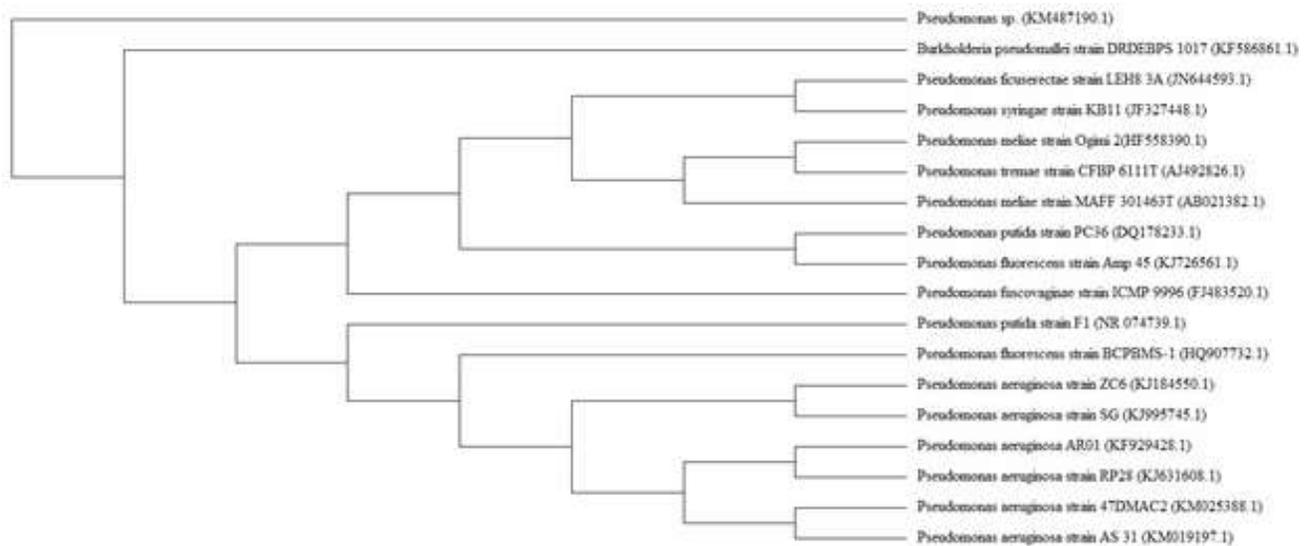


Figure 1. Molecular phylogenetic analysis of *Pseudomonas* sp. strain (KM487190) isolated from rhizosphere soils with some related *Pseudomonas* strains. The evolution history was inferred by using the Maximum Likelihood method. The bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 18 nucleotide sequences (accession numbers in parenthesis). All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted in MEGA6.

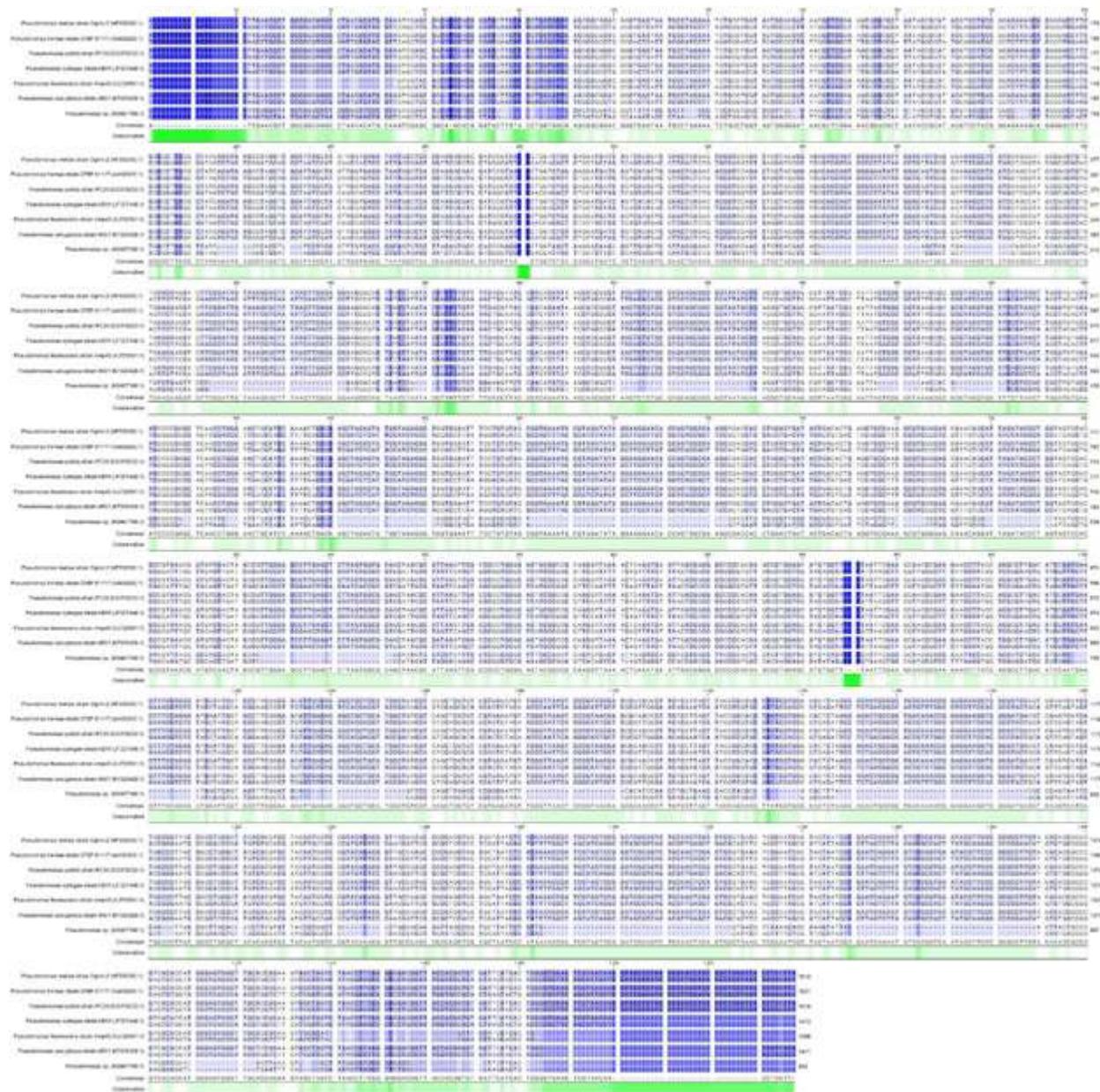


Figure 2: The tabular format of a multiple alignment from seven 16s rRNA genes of *Pseudomonas* strains from the NCBI database using the CLC sequence viewer software, version 7.5. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The level of sequence conservation is shown on a color scale with blue residues being the least conserved and white residues being the most conserved. Besides a color plot (green for the least conserved and white for the maximum ones) shows the conservation extent of each domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2: Inhibitory activity of cell-free culture supernatant (CFCS) and purified proteins (PP) obtained from the isolated *Pseudomonas* sp. against some pathogenic bacteria in comparison with some antibiotics

Pathogens tested	Zone of Inhibition (mm)						
	cell-free culture supernatant	purified protein	TE	CRO	CP	AZM	E
<i>Staphylococcus aureus</i>	17	25	25	23	25	15	20
<i>Escherichia coli</i>	14	15	31	21	25	25	28

Methicillin Resistant <i>Staphylococcus aureus</i>	24	39	27	22	25	20	21
<i>Escherichia coli</i> O15:H7	5	8	20	25	23	24	31

TE: Tetracycline, CRO: Ceftriaxone, CP: Ciprofloxacin, AZM: Azithromycin, E: Erythromycin



Figure 3. Antimicrobial activity of cell-free culture supernatant (CFCS) and purified proteins (PP) obtained from the isolated *Pseudomonas* sp. against 1: Methicillin resistant *Staphylococcus aureus* (MRSA), 2: *Staphylococcus aureus* and 3: *Escherichia coli*

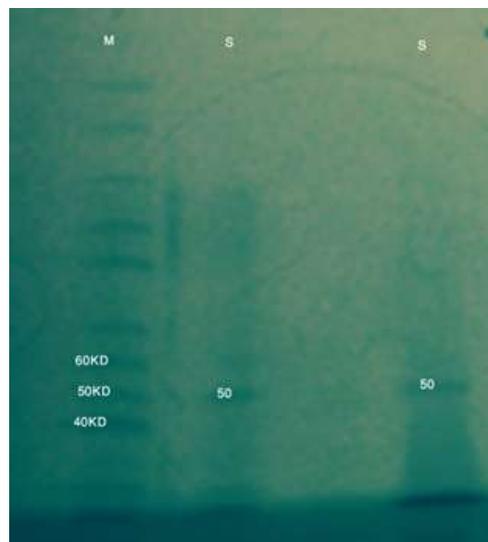


Figure 4. Analysis and identification of the purified antimicrobial substance from *Pseudomonas* sp. The purified antimicrobial substance was analyzed by 15% SDS-PAGE: Gel stained with Coomassie blue G-250: line M, peptide ladder with a molecular mass ranging from 15 to 200 kDa, and line sample, the purified protein

Pseudomonas strains produce different biologically active compounds. There are many reports that note the production of biologically active compounds including different enzymes, phytohormones (such as auxins), and also siderophores by rhizosphere bacteria [26-28]. *Pseudomonas* sp. may have a favorable, neutral or deleterious influence on plant growth [29-31]. Rekha et al [32] have studied the antibacterial activity of *P.*

fluorescens isolated from rhizosphere soils, against ten pathogenic bacterial strains. The results indicated that the naturally isolated *P. fluorescens* strain presented a significant value against *Salmonella typhi*, *B. subtilis*, *Salmonella sonnei*, whilst having no activity against *S. aureus* and *E. coli*. In contrast, the results of present study show that a naturally isolated *Pseudomonas* strain exhibits a significant antibacterial activity against *S.*

aureus, *E.coli* and MRSA. In a similar study performed by Sharma and Kaur [33] some strains of *Pseudomonas* showed a significant antimicrobial activity against most of the tested microorganisms. Besides, the antimicrobial activities of extracellular compounds produced by a *Pseudomonas* strain against MRSA were reported by other researchers [34].

CONCLUSION

Based on this study, we have shown that a *Pseudomonas* sp. strain isolated from the rhizosphere citrus soils have the potential to produce different biologically active compounds. Due to the size of the zone of inhibition it is evident that isolated bacteria have a strong antibacterial agent, especially on gram positives such as MRSA and *S. aureus*. Since molecular weight of the antimicrobial composition is high, likely to be a member of the family of antibiotics that can be investigated in future study. Also, the high inhibition zone on Gram-positive cocci, with the strongest antibiotics standard is similar, that indicates the antibacterial effects of the compound of biology.

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