Isolation and identification of antimicrobial agent-producing bacterium from *Taxus baccata* rhizosphere antagonistic against clinically significant microbes

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Abstract A bacterium identified as *Pseudomonas fluorescence* was isolated from *Taxus baccata* rhizosphere. Ethyl acetate extract from its culture filtrate yielded an active antimicrobial compound that was purified by TLC. The active metabolites were resolved by column chromatography on silica gel (60–120 mesh). The compound was further characterized on the basis of spectral data (UV, IR and ¹H-NMR), which indicated the presence of an aromatic ring and phenolic functionality. The compound showed significant antimicrobial activity against two-gram positive bacteria (*B. subtilis* and *S. aureus*), four-gram negative bacteria (*E. coli*, *K. pneumoniae*, *S. flexneri* and *P. aeruginosa*), and one pathogenic fungus (*Candida albicans*). The minimum inhibitory concentration (MIC) of the compound ranged between 75 µg to 250 µg/ml.

Keywords *Taxus baccata* · *Pseudomonas fluorescence* · antimicrobial activity · Aromatic ring · phenolic functionality

Introduction

Taxus baccata, an evergreen tree of the temperate forests in the Himalayas, has recently assumed tremendous commercial significance because its leaves and bark yield taxol – a proven anticancer metabolite¹. An endophytic fungus *Taxomyces andreanae*, which produces taxol *in vitro*, was isolated from *Taxus* sp.² In addition to this many other endophytes are also isolated from different *Taxus* spp. of the world that produce important metabolites³.

Rhizosphere microbiology has received significant attention during the last century because of the role played by rhizosphere microorganisms in plant growth and health⁴. Fluorescent pseudomonads are ubiquitous bacteria that are common inhabitant of the plant rhizosphere and are the most studied group within the genus *Pseudomonas*. Strains of *Pseudomonas* have shown their abilities to suppress a wide variety of fungal root pathogens in many different agricultural crops around the world^{5–7}. In most cases the biocontrol ability of these strains is directly correlated with the production of antibiotics such as 2-4-diacetylphloroglucinol (DAPG), Phenazines (Phz), Pyrrolnitrin (PRN), Pyoluteorin (PLT) and hydrogen cyanide (HCN)^{8,9}. These antibiotics have been reported to exhibit antifungal, antibacterial, antihelminthic and phytotoxic activity^{10,11}. Most of these antibiotics have been used as a biocontrol agent against phytopathogenic bacteria and fungi¹². Yet little is known about their antimicrobial activity against human pathogenic microorganisms. Only pyrrolnitrin antibiotics produced by some fluorescent pseudomonads have been primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi like *Trichophyton*¹³. The abundance of *P. fluorescence* in the plant rhizosphere is correlated with the importance of their rhizospheric

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competence compared to other microflora. The variability of the environmental conditions of the soil and the host plant can constitute factors of diversification on the evolution and the adaptability of this group of Rhizobacteria. Driven by the appearance of antibiotic-resistant bacterial pathogens, including *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*¹⁴, there is an ever-pressing need for new antibiotics. Similarly, few drugs are available for the treatment of infections caused by yeast (e.g., *Candida albicans* and *Cryptococcus neoformans*) and/or fungi (e.g., *Histoplasma capsulatum* and *Aspergillus niger*)¹⁵. Many compounds such as phenazines, pyoluteorin, pyrrolnitrin, tropolone, pycocyanin and bulbiformin are produced by *Pseudomonas fluorescence* having activity against many bacteria and fungi. Considering specific and intraspecific diversity of fluorescent pseudomonads, the plant and soil type at different areas can play a considerable role in the distribution and taxonomic and metabolic diversity of this group of Rhizobacteria¹⁶. As a result of the increasing

prevalence of antibiotic-resistant pathogens and the pharmacological limitations of antibiotics, there is an exigency for new antimicrobial substances. The results of extensive screening have been the discovery of about 4000 antibiotic substances from bacteria and fungi, many of which have found applications in medicines.

In the present study, the bacterial isolates were isolated from *Taxus baccata* rhizosphere for production of metabolites having potent anti-microbial activity that might facilitate detection of anti-microbial compounds.

Materials and Methods

Soil samples were collected from *T. baccata* rhizosphere from Tawang (27°35'25" N and 91°65'45" E) and Dibang valley (28°20'7" N and 95°46'38" E) district of Arunachal Pradesh, India situated at an altitude of 3250 m and 2600 m respectively. Soil temperature at both the sites was recorded as 17°C. The pH of the soil sample was 6.8. All the samples were collected in sterile polybags and brought into the laboratory. The samples were kept at 4° C till isolation procedure was completed.

The soil had a loamy sand texture and was passed through a sieve to remove the debris. Microorganisms were isolated by dilution plate technique of Johnson and Curl¹⁷. The isolated bacterial strain was grown on nutrient broth media (Himedia). An antagonist bacterial strain showing antimicrobial activity was sent to the Institute of Microbial Technology (IMTECH), Chandigarh, India for identification. Fresh culture of the bacterial strain was inoculated into ten 500 ml Erlenmeyers flasks each containing 250 ml of nutrient broth. The flasks were incubated at 32±1°C for

Fig. 1 Effect of temperature on the production of active metabolite by *Pseudomonas fluorescence* (MTCC 6732).

Fig. 2 Effect of pH on the production of active metabolite by *Pseudomonas fluorescence* (MTCC 6732).

Table 1 Zone of inhibition and minimum inhibitory concentration (MIC) of the metabolite produced by *Pseudomonas fluorescence* (MTCC 6732)

Test Organisms	Zone of inhibition (mm)	Minimum inhibitory concentration (µg/ml)
<i>Bacillus subtilis</i>	25.0±1.0	75±1.5
<i>Staphylococcus aureus</i>	21.8±1.2	75±1.2
<i>Escherichia coli</i>	14.8±0.9	125±0.9
<i>Klebsiella pneumoniae</i>	16.0±0.9	125±1.0
<i>Shigella flexneri</i>	17.8±1.0	125±1.2
<i>Pseudomonas aeruginosa</i>	09.3±1.0	250±1.5
<i>Candida albicans</i>	20.0±0.5	125±1.0

Values are mean of three replicates
± Standard Deviation

Fig. 3 IR Spectrum of the active metabolite. Spectra: IR (ν cm^{-1}) 583.2, 661.3, 704.3, 755.5, 793.8, 836.7, 886.9, 906.2, 1022.0, 1259.3, 1384.6, 1449.0, 1505.1, 1561.3, 1595.2, 1628.5, 1711.9, 2854.7, 2924.6, 3319.4

32 hrs in a rotary shaker at 200 rpm. Activity of the liquid culture was tested against *Bacillus subtilis* (Fig. 6) and *Klebsiella pneumoniae* (Fig. 5) by agar cup diffusion assay method as described by Grammer¹⁸. Different temperatures i.e. 20, 25, 30, 35, 40, and 45°C, incubation periods i.e. 24h, 36h, 48h and 60h, and pH i.e. 4, 5, 6, 7, 8 and 9 were used to observe their effect on the production of metabolites. The culture filtrate was extracted by taking equal volume of ethyl acetate. The organic solvent extract was pooled and then dried with the help of a rotary vacuum evaporator. The crude metabolite obtained was a dark brown pasty mass that was resolved on TLC with eluents of different polarity. The major metabolic product of the bacteria was purified with the help of Column Chromatography using silica gel (60–120 mesh) and 1:2 ethyl acetate and petroleum ether as the eluent. The isolated antimicrobial compound was characterized on the basis of its spectral data obtained using IR and ¹H NMR.

Antimicrobial activity of the compound was tested against seven human pathogenic organisms by agar cup diffusion method. The test organisms include two Gram-positive bacteria, *Bacillus subtilis* (MTCC 441) and *Staphylococcus aureus* (MTCC 96), four Gram-negative bacteria *Klebsiella pneumoniae* (MTCC 619), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* and *Shigella flexneri* (collected from Regional Institute of Medical Science (RIMS), Imphal, India), and a pathogenic fungus *Candida albicans*, obtained from microbiology division of Defense Research Laboratory, Tezpur, India. Nutrient agar plates were inoculated with 0.2ml of overnight culture of each bacterial suspension containing 1.0×10^9 cells. Similarly Sabouraud's agar plate was inoculated with 0.2ml of *C. albicans* containing 1.0×10^9 cells. The plates were evenly spread out with a help of a sterile cotton swab. Agar cups were prepared by scooping out the media with a cork borer (7mm in diameter). The compound was dissolved

in Dimethyl sulfoxide (DMSO) to get a concentration of 1mg/ml. From this 100 μ l were loaded in each cup. The petriplates were incubated at $37\pm 1^\circ\text{C}$ for 24 hrs. The zone of inhibition was recorded and compared with the control (i.e. a cup filled with DMSO solution only). Three replicates were maintained in each case.

The minimal inhibitory concentration (MICs) was determined by micro broth dilution assay as recommended and described by Santos and Rodrigues-Filho¹⁹. The assays were performed in sterile 96-well plates and total volume per well was 100 μ l. The bacterial inoculum was prepared to give approximately 10^4 – 10^5 CFU per well and the compound was tested at concentration from 1000 μ g to 35 μ g/ml in twofold step dilution. Three wells were inoculated for a given concentration. The plates were incubated for 24 hr at $37\pm 1^\circ\text{C}$.

Results and Discussion

Altogether 31 bacteria were isolated from *Taxus baccata* rhizosphere. Out of 31 isolated bacterial species only one, isolate showed good antimicrobial activity against all the test organisms during the preliminary screening test. This potent bacterial strain was identified as *P. fluorescence* with MTCC isolate no. 6732. Different physical parameters such as temperature, incubation time and pH were tested for maximum production of the active metabolites against *Bacillus subtilis* and *Klebsiella pneumoniae*. Optimum production of metabolite required $32\pm 1^\circ\text{C}$ temperature (Fig. 1), 7.5 pH (Fig. 2) and 30h incubation period.

The antimicrobial activity of the major component was determined using seven human pathogenic organisms that include two Gram-positive and four Gram-negative bacteria and one human pathogenic fungus. The results indicated highest zone of inhibition in Gram-positive bacteria, *B. subtilis* (25mm) and least zone of inhibition in Gram-negative bacteria, *P. aeruginosa* (9mm). *C. albicans* showed 20mm zone diameter, which also indicated a good activity. The other test organisms showed moderate zone of inhibition. The compound was tested at a concentration of 1 mg/ml. The minor component was also determined for antimicrobial activity but it showed much lower activity when compared with the major one. The MIC of the compound was determined as 75 μ g/ml against *B. subtilis*, *S. aureus* and *C. albicans* and for *E. coli*, *K. pneumoniae* and *S. flexneri* it was found to be 125 μ g/ml (Table 1). For *P. aeruginosa* it was 250 μ g/ml. The compound was more active against Gram-positive organisms than Gram-negative ones (Table 1). Significant activity of the metabolite against *C. albicans*, a fungal pathogen, indicated a broad-spectrum nature of the

Fig. 4 ^1H NMR of the active metabolite. Spectra: ^1H NMR (δ ppm) 0.829, 1.160, 1.209, 1.461, 2.483, 3.390, 6.307, 6.327, 6.368, 6.957, 6.976, 6.995, 9.461, 13.059

compound. However, *P. aeruginosa* showed lesser activity when compared with the other tested organisms. This may be due the reason that related organisms produce similar compound that may not have much inhibitory effect.

The active compound was recovered by solvent extraction method using ethyl acetate as solvent. The crude metabolites obtained were a brown pasty mass. To ascertain the number of components in the crude product it was subjected to TLC that indicated the presence of a major product with only a minor one that remained static on the base line even

Fig. 5 ^{13}C NMR of the active metabolite. Spectra: ^{13}C NMR (δ ppm) 14.802, 23.000, 29.490, 29.895, 32.182, 39.786, 39.992, 40.198, 40.404, 40.617, 40.823, 41.029, 79.276, 79.604, 79.932, 107.121, 109.958, 115.114, 117.241, 119.987, 130.847, 131.549, 156.282, 183.303

when the polarity of the eluent was considerably enhanced. The R_f values of the major product with the eluent ethyl acetate/petroleum ether in the ratio of 1:1, 1:2 and 1:3 were 0.80, 0.40, and 0.27 respectively. The major component of the crude metabolites was further resolved by column chromatography on silica gel (60–120 mesh) using 1:2 ethyl acetate and petroleum ether as the eluent. Thus 99.7 mg of the crude metabolite yielded 73.4 mg of the active com-

ponent that appeared as a light yellow solid compound. The minor component that appeared on the base line was extracted with ethyl acetate. It weighed only 23.7 mg and was brown pasty mass in appearance. Both the components were examined for their antimicrobial activity. The active compound was further analyzed using IR and ^1H NMR. The IR spectrum of the purified product (Fig. 3 recorded on the sample as KBr pellet with Perkin Elmer FT IR) showed

two strong signals at 2854 and 2924 cm^{-1} indicating the presence of an aromatic ring. A band at 3319 cm^{-1} indicated the presence of either N-H or O-H group. Signal between 6 to 7 ppm in the ^1H NMR (Fig. 4 recorded with Varian 400 MHz FTNMR using $\text{DMSO-d}_6 + \text{CDCl}_3$ as solvent and TMS as internal reference) indicated the presence of aromatic proton. The ^{13}C NMR spectrum (Fig. 5) of the same sample indicates the presence of not less than ten different carbon atoms. The sharp singlet at 9.5 ppm indicates the presence of phenolic functionality (Fig. 4). The ^1H NMR signal along with the IR band at 3319 cm^{-1} gave an impression that the major component may be a phenolic one. The sharp singlet counting nearly 9 protons at 3.4 ppm gives an indication of the presence of an O-alkyl group that is probably a *tert*-butyl one. Although the compound has been partially characterized, however, based on the present spectral data (IR, ^1H -NMR and ^{13}C NMR) it is characterized as aromatic and phenolic functionality which indicates that it could be phloroglucinol antibiotic. Efforts are on for further characterization and structure elucidation of the active metabolites.

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