J. Microbiol. Biotechnol. (2009), **19**(12), 1688–1694
doi: 10.4014/jmb.0904.04022
First published online 26 September 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of a Phenazine and Hexanoyl Homoserine Lactone Producing *Pseudomonas aurantiaca* Strain PB-St2, Isolated from Sugarcane Stem

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Received: April 15, 2009 / Revised: July 17, 2009 / Accepted: July 28, 2009

A novel strain of fluorescent pseudomonad (PB-St2) was isolated from surface-sterilized stems of sugarcane grown in Pakistan. The bacterium was identified as *Pseudomonas aurantiaca* on the basis of 16S rRNA gene sequence analysis and results from physiological and biochemical characteristics carried out with API50 CH and QTS 24 bacterial identification kits. Assays using substrate-specific media for enzymes revealed lipase and protease activities but cellulase, chitinase, or pectinase were not detected. The bacterium was unable to solubilize phosphate or produce indole acetic acid. However, it did produce HCN, siderophores, and homoserine lactones. In dual culture assays on agar, the bacterium showed antifungal activity against an important pathogen of sugarcane in Pakistan, namely *Colletotrichum falcatum*, as well as for pathogenic isolates of *Fusarium oxysporium* and *F. lateritium* but not against *F. solani*. The antifungal metabolites were identified using thin-layer chromatography, UV spectra, and MALDI–TOFF spectra and shown to be phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine (2-OH-PHZ), and *N*-hexanoyl homoserine lactone (HHL) (assessed using only TLC data). The capacity of this bacterium to produce HCN and 2-OH-PHZ, as well as to inhibit the growth of *C. falcatum*, has not been previously reported.

Keywords: *Pseudomonas aurantiaca*, phenazine-1-carboxylic acid, 2-hydroxyphenazine, *N*-hexanoyl homoserine lactone, *Colletotrichum falcatum*, sugarcane

Sugarcane is an important cash crop grown over one million hectares in Pakistan. Although production continues to increase, there has been only a minimal increase in yield per unit area. A major contributor to yield losses is the widely distributed red-rot disease induced by the fungus *Colletotrichum falcatum*. This disease is responsible for the deterioration of sugarcane stands and is also a problem in other countries such as the U.S.A., Australia, Taiwan, Thailand, India, and Bangladesh. There is no known economically feasible protective measure that one can take to limit the impact of this disease. Most farmers try to use disease-free seed canes for planting, but generally such measures are impractical, as dormant fungal infections are common and not readily diagnosed. The use of biological control agents has been suggested as a potential option for disease control, but as yet there has not been any research undertaken in Pakistan to identify potential biocontrol agents.

Fluorescent pseudomonads have received the most prominent attention as candidates for biocontrol agents because of their ability to colonize the surfaces and internal tissues of roots and stems (endo- and exorhizosphere) at high densities. These bacteria can compete successfully with soil microorganisms and have tremendous capacity for production of antifungal secondary metabolites. Over 20 *Pseudomonas* species are known to synthesize more than 100 aromatic antibiotics and antibiotic-like compounds [4]. Some well-known antibiotics are phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), 2,4-diacetyl phloroglucinol (Phl), pyocyanin, 2-acetamidophenol, pyrolnitrin, pyoluteorin, viscosinamide, and tesin [9]. A number of *Pseudomonas*-based biocontrol inoculants have now been commercially developed [13].

The objective of the current study was to isolate and screen the most promising bacterial strain for the suppression of *Colletotricum falcatum*, associated with red-rot disease of sugarcane. For this purpose, we sampled indigenous varieties of sugarcane grown in the vicinity of five cities of Punjab. Bacterial strains were isolated from the roots,

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shoots, and rhizosphere soil of sugarcane. After screening many isolates for antifungal activity against pathogenic strains of *C. falcatum*, we identified one strain with uniquely potent antifungal activity against this and other fungal pathogens. Assessment of metabolites such as antibiotics, siderophores, homoserine lactones, HCN, and enzymes like protease, cellulase, and lipase, which may be involved in the mechanisms of biocontrol, was also conducted.

Materials and Methods

Isolation and Characterization of PB-St2

Strain PB-St2 was isolated from the stem of an indigenous sugarcane variety, CPF 240, collected from Pindi Bhattiyan, Punjab. Isolation was done in semisolid LGI medium [1] and bacterial culture was maintained on LGI and King's B media [7]. For identification of PB-St2, study of cell and colony morphologies, biochemical tests, and analysis of the gene sequence encoding 16S rRNA were carried out.

Cell and Colony Morphologies

Colony morphology was observed on LGI and KB plates and cell morphology was observed using the transmission electron microscope Zeiss EM902.

Biochemical Tests

Biochemical tests were performed by using API50 CH (BioMerieux, Marcy l'Etoyle, France) and QTS24 (Desto Laboratories, Karachi, Pakistan) bacterial identification kits. Catalase was identified by the MacFadden [10] method using H_2O_2 and pure culture colonies from agar plates. Bacterial growth at different temperatures (4–41°C), pH (5–12), and various NaCl concentrations (0.5–5%) was determined on LB medium.

PCR Amplification, 16S rDNA Sequence Analysis, and Phylogenetic Tree

A single colony of PB-St2 was inoculated into LB broth, and after overnight growth at 30°C, the DNA from the bacterial cells was isolated by using the QIAGEN blood and cell culture DNA Midi kit. The DNA was dissolved in 100 µl of TE buffer and used as a template for PCR amplification of 16S rDNA. The amplification primers and PCR conditions were the same as previously described by Mehnaz *et al*. [15]. Each reaction mixture (50 µl) contained 0.5 µl of *Taq* polymerase (5 U/µl), 5 µl of PCR buffer, 2.5 µl of $MgCl_2$ (50 mM), 1 µl of dNTPs (10 mM), 1 µl (10 µM) of each primer (FGPS4-281 and FGPS1509-153), 37 µl of filter-sterilized milli Q water, and 2 µl of template DNA. The PCR product was purified by using a QIAquick PCR purification kit (QIAGEN), cloned in Promega PGEM T-easy vector, and sequenced on an Applied Biosystems 3730 analyzer. The sequence was deposited in GenBank (Accession No. EU761590).

An alignment of nucleotide sequences of the isolate PB-St2 and related *Pseudomonas* strains was carried out by using Clustal W (version 1.83). The evolutionary history was inferred using the Neighbor-Joining method [26]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches [5]. The evolutionary distances were computed using the Maximum Composite Likelihood method [28] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There was a total of 1,423 positions in the final dataset. Phylogenetic analysis was conducted in MEGA4 software [29].

Antibiotics Resistance Pattern

An antibiotic resistance pattern was identified for ampicillin, chloramphenicol, gentamicin, kanamycin, rifampicin, spectinomycin, streptomycin, and tetracycline, from 25 to 100 µg/ml, on LB plates.

Detection of Different Enzymes

Phosphatase and phytase activities were checked on NBRIP [17] and calcium phytate agar media [24], respectively. Cellulase and chitinase activities were tested on LB plates, with the respective substrate added (carboxymethyl-cellulose 1%, chitin 0.5%). Pectinase and protease activities were checked on the media described by Kumar *et al*. [8]. Lipase production was identified on the medium described by Rashid *et al*. [23]. Aliquots of bacterial culture (10 µl), grown overnight in LB broth, were spot-inoculated onto plates. Plates were incubated for 2–8 days at 30°C and formation of a clear zone around the bacterial growth spot was considered as a positive result for enzyme activity.

Indole Acetic Acid (IAA) Production

A bacterial culture was grown for 7 days, in rich medium (g/l; glucose, 2.0; glutamic acid, 1.5; peptone, 1.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 2.0; pH 6) and KB medium. L-Tryptophan (100 mg/l) was then added to both media as a precursor of IAA biosynthesis. Cells were pelleted at 10,000 rpm and the supernatants used for detection and quantification of IAA by using a colorimetric method [6]. Standard solutions were prepared with pure indole-3-acetic acid (Sigma) for quantification of IAA produced by a bacterial strain.

Hydrocyanic Acid Production

To qualitatively determine the hydrocyanic acid (HCN) production, the bacterial strain was streaked onto KB medium, and filter paper saturated with an alkaline picrate solution (g/l: picric acid, 2.5; Na_2CO_3, 12.5; pH 13) was placed into the lid of the petri plate containing the culture [16]. The petri plate was then sealed with parafilm and incubated for four days. HCN production was assessed by the change of filter paper from yellow to brown/reddish brown.

Siderophore Production

Siderophore production was detected using the O-CAS method [22]. The pellet obtained from an overnight grown bacterial culture was resuspended in sterilized water, and 10 µl of this suspension was then spotted onto LB plates and incubated at 30°C for 48 h. CAS medium was prepared according to Schwyn and Neilands [27]. Ten ml of this medium was used as overlay and applied to the LB agar plate containing 48-h grown bacterial culture. A change in color from blue to orange (hydroxamate-type siderophore) or purple (catechol-type siderophore) was considered as a positive reaction. Sterilized LB and water were used as negative controls.

Detection and Extraction of Acyl Homoserine Lactones (AHL)

C. violaceum CV026 was streaked on LB plate containing 25 µg/ml kanamycin. After overnight growth at 28°C, cells were transferred to

sterile water using a sterile loop and the OD was adjusted to 0.4 at 600 nm. Five ml of this bacterial suspension was added to 200 ml of cooled TY (g/l, tryptone, 5.0; yeast extract, 3.0; $CaCl_2$ 1.11) agar and the agar was poured into plates. A single colony of PB-St2 was inoculated into 5 ml of LB medium and grown for 24 h. *R. leguminosarum* pRL1j1 was individually grown in 5 ml of TY for 24 h. Aliquots 10 µl of PB-St2 and *R. leguminosarum* pRL1j1 were spotted on agar plates containing CV026 and air dried. Plates were incubated for 2–3 days at 28°C. Quorum-sensing was detected by the appearance of a violet halo around the colony if violacein production occurred as a result of the activation of the reporter gene in *C. violaceum*. *R. leguminosarum* pRL1j1 was used as positive control. TY and LB media were used as negative controls. *C. violaceum* CV026 and *R. leguminosarum* pRL1j1 were kindly provided by Allen Downie, John Innes Centre, Norwich, U.K.

AHL was extracted twice from a cell-free supernatant using dichloromethane (2.5:1). Residual water in the extract was removed by addition of anhydrous sodium sulfate. The extract was evaporated to dryness and the residue was redissolved in ethyl acetate. A 10-µl sample was used to perform thin-layer chromatography (TLC) on a silica gel plate. Synthetic *N*-hexanoyl L-homoserine lactone (HHL; Sigma) was used as the standard (10.0 ng). The TLC plate was developed with methanol:water [90:10 (v/v)]. The presence of AHL was detected by overlaying the TLC plate with an exponentially grown culture of *C. violaceum* CV026 [14].

Antifungal Activity

The antifungal activity of PB-St2 was examined using four strains of fungal pathogens; *Colletotrichum falcatum,* isolated from local sugarcane varieties (BF166, C01148, CP77400, SPF234) and three isolates of *Fusarium* spp. (*F. solani*, *F. oxysporium*, *F. lateritium). C. falcatum* strains were kindly provided by Shukur Gunj Research Institute, Jhang, Pakistan, and *Fusarium* isolates were purchased from Fungal Culture Bank, Lahore, Pakistan. Potato dextrose agar (PDA; Difco, MI, U.S.A.) was used for these assays. A small plug cut from a growing edge of the mycelium of the target fungus was seeded at the center of the medium in a petri plate. Aliquots (10 µl) of bacterial cultures grown in LB medium overnight were spotted 2 cm away from the center (one isolate per plate) and air dried in a laminar flow cabinet before incubation. Plates were incubated for 10–15 days at 25°C. Antifungal activity was observed by formation of a zone of inhibition of mycelial growth.

Extraction and Identification of Antifungal Metabolites

A bacterial culture was grown in 200 ml of LB broth for 90 h at 30°C. The culture was harvested at 28,000 rpm for 30 min. The pH of the cell-free supernatant was adjusted to 9 with 1 N NaOH and extracted with 30 ml of chloroform. The pH of the supernatant was then adjusted to 3.0 with 1 N HCl and extracted four times with a total of 120 ml of chloroform (30 ml chloroform/extraction). The organic fraction was washed with water, dehydrated with anhydrous sodium sulfate, and evaporated to dryness. The residue was redissolved in 500 µl of methanol. A 20 µl sample was applied to a silica gel plate and chromatography was carried out in chloroform:acetic acid (49:1; v/v). The silica containing visibly colored yellow and orange spots was carefully removed from the plate and the compounds it contained were eluted using 20 µl of methanol. The silica was removed using centrifugation, and 10 µl of the clear supernatant was used to check for antifungal activity. The rest of this material was run on a TLC plate using the same solvent system as previously, described to check the purity of the compounds. UV spectra of the repurified compounds were scanned on a UV-2405 spectrophotometer (Shimadzu, Japan). The mass of the compounds were determined by a MALDI–TOF (Autoflex III Smartbeam 200 System) vertical mass spectrometer (Bruker Daltonics, Germany). Samples were analyzed in α-cyano-4-hydroxycinnamic acid (Matrix A; Sigma), where 5 µl of purified compounds was individually mixed with 5 µl of Matrix A (suspended in 0.1% trifluoric acid in water:acetonitrile; 2:1; v/v). Then, 2 µl of this solution was loaded on a MALDI–TOF plate. The samples were air dried at room temperature, before analysis.

RESULTS

Cell and Colony Morphologies

Isolate PB-St2 formed 1–5 mm diameter, yellow, round, convex, wax-drop-like colonies, with entire margin on LGI medium. On King's B medium, PB-St2 formed 1–3 mm, round, flat, deep orange colonies that stayed orange while the medium stained green and fluoresced under UV light. Cells of the bacterium are rods, 0.8–1.2 µm wide and 1.6–3.6 µm long, motile, and lophotrichous, with four to six flagella (Fig. 1).

Biochemical Tests

The isolate utilized glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-melibiose, D-mannitol, D-saccharose, D-trehalose, D-raffinose, D-lyxose, D-fucose, D-arabitol, inositol, gentiobiose, potassium gluconate, esculin ferric citrate, and *N*-acetyl glucosamine, but did not utilize erythritol, dulcitol, D-mannose, D-adonitol, L-sorbose, D-sorbitol, methyl beta D-xylopyranoside, methyl alpha D-manno pyranoside, methyl alpha D-glucopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-lactose, inulin, D-melezitose, amidon, glycogen, xylitol, D-turanose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, and potassium 5-ketogluconate as a sole carbon source. The bacterium produced acid from D-glucose, D-mannitol, and L-arabinose, but not from L-rhamnose, D-sorbitol, inositol, D-adonitol, D-melibiose, and

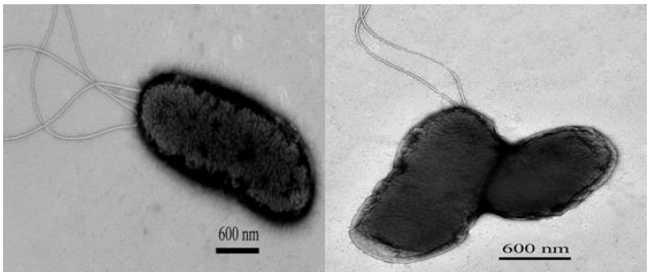

Fig. 1. Transmission electron micrograph of sugarcane isolate PB-St2.
(Magnification=×12,000)

D-raffinose. Isolate PB-St2 is positive for arginine dihydrolase, catalase, cytochrome oxidase, trisodium citrate, gelatin hydrolysis, and 2-nitrophenyl-β-D-galactopyranoside, and negative for acetoin, indole production, nitrate reduction, lysine decarboxylase, 2-nitrophenyl-β-D-galactopyranoside, ornithine decarboxylase, sodium thiosulfate, tryptophane deaminase, and urease. Bacterial growth was observed at 4 to 37°C, pH 5 to 10, and up to 3% NaCl concentration.

PCR Amplification and 16S rDNA Sequence Analysis

An almost complete sequence (~1.5 kb) of 16S rDNA was obtained for isolate PB-St2 and compared with the NCBI databank through a Blast search. The isolate showed 99.8% similarity (1,524/1,527 positions) with the sequence of *P. aurantiaca* ATCC 33663^T (Accession No. DQ682655) and 99.5% similarity (1,521 identities/1,529 positions: 1,485 identities/1,492 positions) with the sequences of *P. aureofaciens* ATCC 13985^T (Accession No. AY509898) and *P. chlororaphis* ATCC 9446^T (Accession No. Z76673), respectively. The phylogenetic tree is shown in Fig. 2.

Antibiotics Resistance Pattern

PB-St2 is resistant to ampicillin, chloramphenicol, and spectinomycin at 100 μg/ml, streptomycin at 75 μg/ml, and tetracycline at 25 μg/ml and sensitive to rifampicin, kanamycin, and gentamicin at 25 μg/ml.

Detection of Different Enzymes, IAA, HCN, and Siderophore Production

The bacterium showed positive reactions for protease and lipase and was negative for the production of cellulase, chitinase, pectinase, phosphatase, and phytase. It did not produce indole acetic acid in both media, but did produce HCN and hydroxamate-type siderophores.

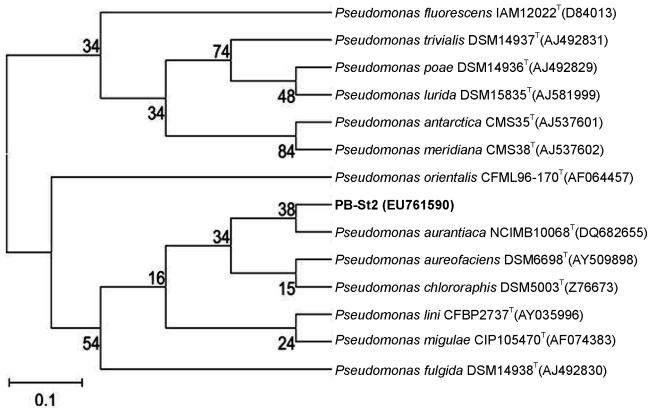

Fig. 2. Neighbor-joining tree of the 16S rRNA gene sequence of bacterial isolate PB-St2.

Sequences of type strains were obtained from databases and accession numbers are in parentheses. The isolate from sugarcane is in bold letters. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

Fig. 3. Thin-layer chromatogram of *N*-acyl-homoserine lactone (AHL), present in cell-free supernatant of *P. aurantiaca* PB-St2 detected using a *C. violaceum* CV026 overlay.

Synthetic AHL and cell-free supernatant from PB-St2 (extracted with dichloromethane) were spotted on a TLC plate and chromatographed as described in Materials and Methods. Lane 1, *N*-hexanoyl homoserine lactone standard (HHL); Lane 2, *P. aurantiaca* PB-St2 supernatant extract.

Quorum Sensing and Antifungal Activity

The isolate was positive for quorum-sensing signal production. On TLC plate, only one purple spot was detected and the compound was identified as HHL (Fig. 3). Antifungal activity was observed against all strains of *C. falcatum, F. oxysporium*, and *F. lateritium*, but not for *F. solani*. Two colored spots were clearly visible on TLC plate, without UV (Fig. 4.), and after purification, both compounds showed antifungal activity against all strains of *C. falcatum, F. oxysporium*, and *F. lateritium.* The purified bright yellow spot showed maximum UV adsorption at 250 and 362 nm in methanol, and the deep orange spot showed maximum UV absorption at 254 and 358 nm (Fig. 5A and 5B). These compounds were identified as phenazine-1-carboxylic

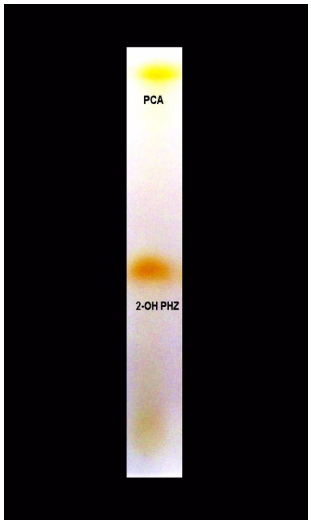

Fig. 4. Thin-layer chromatogram of phenazines, present in cell-free supernatant of *P. aurantiaca* PB-St2.

Cell-free supernatant was acidified, extracted with chloroform, spotted on TLC plate, and chromatographed as described in Materials and Methods. Phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ) were detected as bright yellow and orange spots on TLC plate.

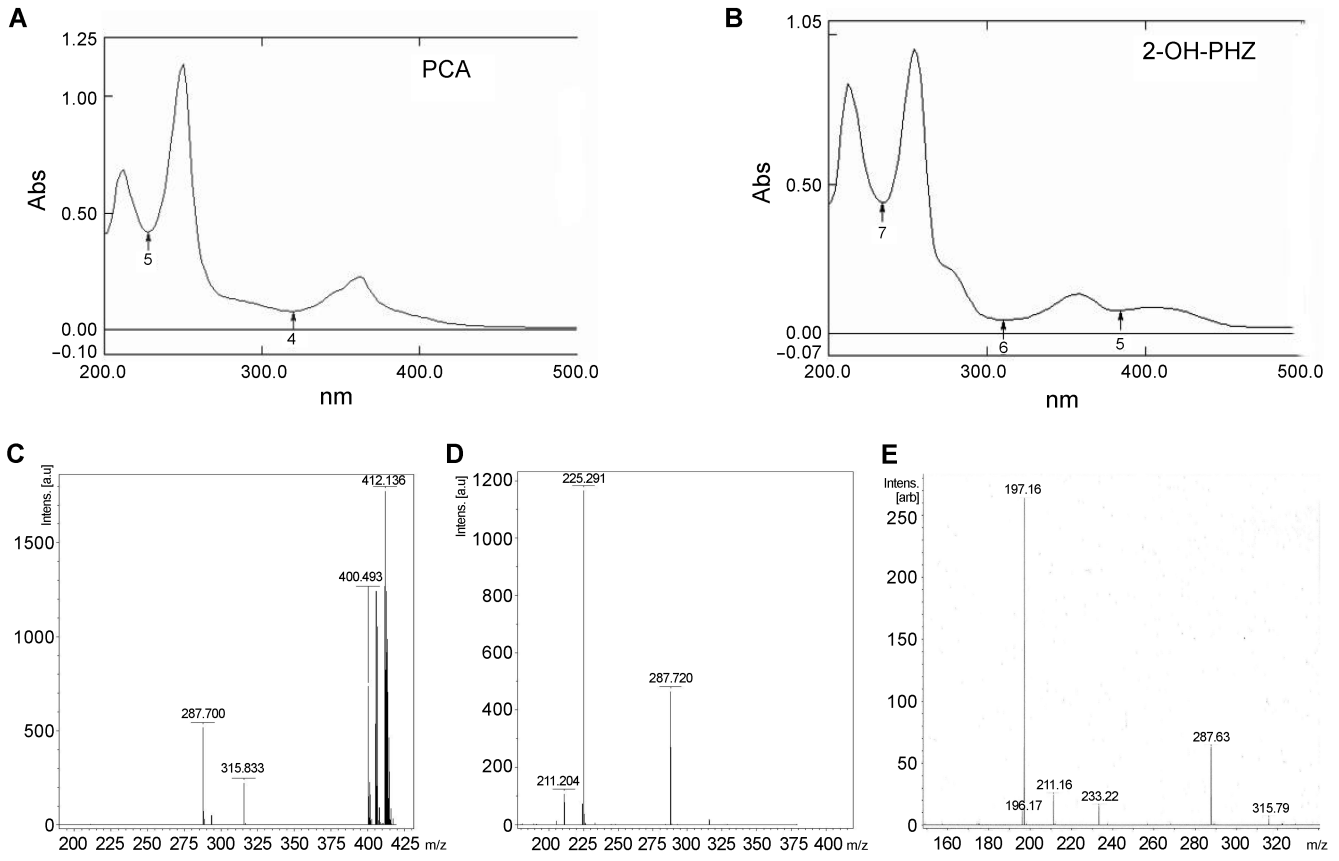

Fig. 5. The ultraviolet and mass spectra of phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ), extracted from cell-free supernatant of *P. aurantiaca* PB-St2.

These compounds were isolated and purified by TLC as described in Materials and Methods. For mass spectrometery, samples were analyzed in α-cyano-4-hydroxycinnamic acid (Matrix A) where 5 µl of purified compounds was individually mixed with 5 µl of Matrix A. UV spectra of PCA (**A**) and 2-OH-PHZ (**B**), at 200–500 nm; Mass spectrum of "matrix A" (**C**); Mass spectrum of "matrix A" spiked with PCA (**D**); Mass spectrum of "matrix A" spiked with 2-OH-PHZ (**E**).

acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ), as peaks on MALDI–TOFF were observed at *m/z* 225 and 197, consistent with PCA and 2-OH-PHZ (Fig. 5D and 5E).

DISCUSSION

Among PGPR, fluorescent pseudomonads occur commonly in the rhizosphere of plants and they are an important functional group of beneficial bacteria for the control of soilborne plant pathogens [3]. The aim of the present study was the screening and selection of rhizobacteria indigenous to sugarcane plants with antagonistic activity against the *C. falcatum* associated with red-rot of sugarcane. Isolation of the bacteria from the target crop is essential for successful identification of potential biocontrol agents [30]. In this report, we described the characterization of a newly isolated strain, PB-St2, of *P. aurantiaca* and its major secondary metabolites. This strain showed antifungal activity against local strains of *C. falcatum* and *Fusarium* spp., phytopathogens of agricultural significance. Two main antibiotics were isolated and identified to be phenazine-1-carboxylic acid and 2-hydroxyphenazine. We also identified the production of *N*-hexanoyl L-homoserine lactone, a quorum-sensing-signal producing compound, by this bacterium in LB medium. Strain PB-St2 was able to produce HCN and siderophores, as PGPR traits. All of these data suggest that PB-St2 could be used as a potential effective biocontrol agent and a biofertilizer, to decrease the incidence of plant diseases and promote the plant growth.

Earlier studies have reported the production of various antifungal compounds including phenazine and its derivatives (*i.e.*, 1-oxyphenazine, phenazine-1,6,dicarboxylate, and 2-hexyl-5-propyl-alkylresorcinol) by *P. aurantiaca* [4, 18]. Recently, Mandryk *et al*. [12] reported new antimicrobial compounds produced by this bacteria, having molecular formulas of $C_{18}H_{36}NO$ (molecular mass 282.8; antibacterial activity) and $C_{20}H_{31}O_3$ (molecular mass 319.3; antifungal activity). Reports about PCA production by *P. aurantiaca*

could not be found, although Peix *et al*. [20] used the production of phenazine-1-carboxylate as a characteristic feature of *P. aureofaciens* and *P. aurantiaca* when they reclassified these two bacteria as subspecies of *P. chlororaphis.* Similarly, 2-OH-PHZ and HCN production by *P. aurantiaca* have not been reported. However, siderophore production in *P. aurantiaca* is reported by Rovera *et al*. [25].

A prerequirement for bacterial growth promoting effect, as a biofungicide or biofertilizer, is the close contact between the plant and the effective organism. PB-St2 inhibited the growth of *C. falcatum* strains, which are isolated from local sugarcane varieties. *C. falcatum* affects the plant stem, and the isolation of PB-St2 from the inner region of the stem supports not only its survival within tissue but also its potential to be used as a biocontrol agent. Inhibition of *C. falcatum* was reported earlier by *Pseudomonas aeruginosa* [8] and *P. fluorescens* [11] but not by *P. aurantiaca*.

Quorum sensing is the major mechanism by which many bacteria regulate production of antifungal factors. PB-St2 produces *N*-hexanoyl homoserine lectone (HHL), a chemical that indicates the presence of a quorum-sensing mechanism. Feklistova and Maksimova [4] previously reported the production of HHL by *P. aurantiaca* B-162. Pearson *et al*. [21] and Chin-A-Woeng *et al*. [2] have shown that quorum sensing is the most important regulation mechanism for PCA production in the closely related species to *P. aurantiaca* B-162, namely *P. aeruginosa*, and for phenazine-1-carboxamide production in *P. chlororaphis*.

Several *Pseudomonas* strains have already been marketed as commercial biocontrol products, such as Cedomon (BioAgri AB, Upsala, Sweden), a seed treatment based on a *Pseudomonas chlororaphis* strain providing protection against seedborne diseases in barley. Similarly, Mycolytin is an antifungal biopesticide formed by *P. aurantiaca* M-518 [19]. The reports in the literature and the presence of biocontrol traits (siderophore, HCN, PCA, and 2-OH-PHZ production) in this strain enhance the potential use of PB-St2 as an effective biocontrol agent promoting plant growth with reduced disease incidence. Future studies will focus on its use as a biocontrol agent in pot and field experiments.

In this study, for the first time, we report about PB-St2 as a new strain of *P. aurantiaca* from the sugarcane stem, with the production of siderophores, HCN, PCA, 2-OH PHZ, lipase, and protease.

Acknowledgments

We are thankful to Ann Fook Yang, and the EM Unit of Eastern Cereal and Oil-Seed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada. We are thankful to Dr. M. Akhter, Director General, School of Biological Sciences, University of the Punjab, Lahore, Pakistan, for his guidance in chromatographic analysis.

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