Antimicrobial activity of bacteria associated with the rhizosphere and phyllosphere of *Avena fatua* and *Brachiaria reptans*

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Abstract

Environmental pollution especially heavy metal-contaminated soils adversely affects the microbial communities associated with the rhizosphere and phyllosphere of plants growing in these areas. In the current study, we identified and characterized the rhizospheric and phyllospheric bacterial strains from *Avena fatua* and *Brachiaria reptans* with the potential for antimicrobial activity and heavy metal resistance. A total of 18 bacterial strains from the rhizosphere and phyllosphere of *A. fatua* and 19 bacterial strains from the rhizosphere and phyllosphere of *B. reptans* were identified based on 16S rRNA sequence analysis. Bacterial genera, including *Bacillus*, *Staphylococcus*, *Pseudomonas*, and *Enterobacter* were dominant in the rhizosphere and phyllosphere of *A. fatua* and *Bacillus*, *Marinobacter*, *Pseudomonas*, *Enterobacter*, and *Kocuria*, were the dominating bacterial genera from the rhizosphere and phyllosphere of *B. reptans*. Most of the bacterial strains were resistant to heavy metals (Cd, Pb, and Cr) and showed antimicrobial activity against different pathogenic bacterial strains. The whole-genome sequence analysis of *Pseudomonas putida* BR-PH17, a strain isolated from the phyllosphere of *B. reptans*, was performed by using the Illumina sequencing approach. The BR-PH17 genome contained a chromosome with a size of 5774330 bp and a plasmid DNA with 80360 bp. In this genome, about 5368 predicted protein-coding sequences with 5539 total genes, 22 rRNAs, and 75 tRNA genes were identified. Functional analysis of chromosomal and plasmid DNA revealed a variety of enzymes and proteins involved in antibiotic resistance and biodegradation of complex organic pollutants. These results indicated that bacterial strains identified in this study could be utilized for bioremediation of heavy metal-contaminated soils and as a novel source of antimicrobial drugs.

Keywords Bacterial diversity · Antibiotic and heavy metal resistance · *Avena fatua* · *Brachiaria reptans* · *Pseudomonas putida*

Introduction

The increasing industrialization mainly affects the quality of water, air, and soil. Anthropogenic activities over the past decades, including pollution with heavy metals, pesticides, and chemical fertilizer, as well as improper soil exploitation coupled with climate change, have resulted in immense global soil degradation (Smith et al., 2015). Environmental challenges of Pakistan are primarily associated with an imbalanced economic and social development in recent decades. Industrial effluents contain hazardous chemicals including heavy metals, highly acidic, and alkaline compounds that cause the destruction of habitat (Freije 2015; Mohmand et al. 2015). Heavy metals, such as cadmium (Cd), lead (Pb), chromium (Cr), and arsenic (As), cause groundwater and soil pollution and ultimately enter the plants and animals through the food chain. Living organisms including plants, animals, and microorganisms that exist in industrial areas are also affected by water, soil, and air pollution (Wahid et al. 1995; Khan et al. 2013; Kamal et al. 2014). In the industrial areas of Pakistan, the growth of natural vegetation including grasses, *Avena fatua*, *Cymbopogon jwarancusa*, *Brachiaria reptans*, and *Cynodon dactylon* as well as economically important crops, such as wheat, rice, maize, and sugarcane are affected due to poor water and air quality (Hussain et al. 1992; Khan et al. 2011; Waseem et al. 2014).

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Plant physiology and metabolism are greatly affected by microorganisms living in the plant soil (rhizosphere), on the surface of a root or shoot (epiphytes), and inside the root or shoot tissues (endosphere). Plant-associated microbial communities include plant growth-promoting bacteria, fungi, and other pathogenic microorganisms (Smith et al. 2015; Mukhtar et al. 2019a). A large variety of microorganisms live in the rhizosphere and root endosphere that have the potential to enhance plant growth by increasing the uptake to different nutrients, nitrogen, carbon, phosphorus, and other minerals from the soil (Ahemad and Kibret 2014; Kuan et al. 2016; Mukhtar et al. 2019c). The aerial portion or phyllosphere of a plant is directly affected by different air pollutants, changes in temperature, humidity, and solar radiations, and is considered as a nutrient-limited environment as compared to the rhizosphere, so the microbial diversity associated with the phyllosphere is distinct. Proteobacteria, Actinobacteria, and Bacteroidetes are the most abundant bacterial phyla identified from this plant region (Bodenhausen et al. 2013; Mukhtar et al. 2017). These microorganisms play an important in plant health and geochemical cycles of nitrogen and carbon (Mazinani et al. 2017).

Plant-associated microorganisms produce a variety of antibacterial and antifungal compounds that play a crucial role to control different bacterial and fungal pathogens (Knief et al. 2012; Sun et al. 2017). Microbial communities associated with the plants growing in polluted areas are also influenced by the different hazardous compounds and heavy metals present in the surrounding environment (Hong et al., 2011; Suvega and Arunkumar 2014). Microbial diversity identified from the rhizosphere of *Broussonetia papyrifera* and *Ligustrum lucidum* growing in heavy metal-contaminated soils was significantly higher and different as compared to the microbial diversity identified from non-polluted soils (Guo et al. 2019). These microorganisms show antimicrobial activity against a large number of pathogenic bacteria and fungi. The rhizosphere- and phyllosphere-associated bacterial strains including *Bacillus*, *Pseudomonas*, *Aeromonas*, *Marinobacter*, *Nocardia*, *Sphingomonas*, and *Methylobacterium* have been studied for their ability to produce various antimicrobial compounds (Bodenhausen et al. 2013; Buedenbender et al. 2017; Chen et al. 2019). These bacterial strains contain plasmids that play a role in the adaptation of various abiotic stresses. Microbial plasmids encode genes for different traits such as antibiotic and heavy metal resistance, osmoregulation, virulence, plant tumors, root nodulation, and nitrogen fixation (Ismail et al. 2016; Zhao et al. 2018).

Some plants are genetically adapted to grow and reproduce in soils contaminated with heavy metals. Plants species such as *Avena fatua*, *Brachiaria reptans*, *Cynodon dactylon*, and *Dactyloctenium aegyptium* are dominantly growing in heavy metal-polluted lands near Lahore (Ahmad et al. 2009).

Environmental pollution also influences the structure and composition of rhizospheric and phyllospheric microbial communities of the affected plants. The main aim of the current study was to evaluate the bacterial diversity from the rhizosphere and phyllosphere of *Avena fatua* and *Brachiaria reptans* growing in heavy metal-polluted areas near Lahore by using culture-dependent techniques. We predicted that we would find bacteria with antibiotics and heavy metal resistance. These bacterial strains would have antimicrobial potential against different bacterial pathogens. The current study is the first report of its kind that deals with the identification of antimicrobial and heavy metal resistance genes from *P. putida* BR-PH17 isolated from the phyllosphere of *B. reptans* through whole-genome sequence analysis.

Material and methods

Soil and plant sampling

Kala-Shah Kaku is an industrial area located on Lahore-Gujranwala G.T road about 17.5 km away from Lahore (31° 24′ north latitude, 74° 13′ east longitude). It covers about 11 km^2 area with a number of industries involved in the production of chemical, leather, textile, metals, paper and pulp. Groundwater and air quality is adversely affected by different untreated effluents, such as heavy metals Cd, Pb, Cr, and As released by these industries. Plants and animals of this area are also affected by water and air pollution. Plants especially various grasses, such as *Avena fatua*, *Brachiaria reptans*, *Cymbopogon jwarancusa*, *Cynodon dactylon*, and *Dactyloctenium aegyptium* are dominant and abundantly found here (Ahmad et al. 2009). Rhizospheric soil samples were collected by gently removing the plants and obtaining the soil (≈ 5 mm) attached to the roots. Soil and plant samples were collected from three sites that are about 1 km far from each other. At each site, approximately 1-kg soil samples were collected in black sterile polythene bags. These samples were stored at 4 °C for further analysis.

Soil physicochemical parameters

Each soil sample (500 g) was thoroughly mixed and sieved through a pore size of 2 mm. Physical properties (salinity, pH, moisture content, and temperature) of soil samples were determined. Soil salinity or electrical conductivity (dS/m) was measured by 1:1 (w/v) soil to water mixture at 25 °C (Adviento-Borbe et al. 2006), pH was measured by 1:2 (w/v) soil to water mixture, moisture (%) and texture class were measured by Anderson method (Anderson and Ingram 1993), and organic matter (C_{org}) was calculated by the Walkley and Black (1934). The total concentration of heavy metals (Cd, Pb, and Cr) in the soils and plants were

analyzed by flame atomic absorption spectrophotometry (AAS, Z-5300) by digesting 100 mg of soil in a mixture of HNO3 and HClO4 (4:1, v/v).

Isolation of bacterial strains from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans*

Rhizospheric samples were taken as a collective sample of rhizosphere and root endosphere and phyllospheric samples were taken as epiphytic and endophytic shoot tissues. For the isolation of rhizospheric bacteria, the sieved soil and roots were mixed thoroughly, and then 1 g representative soil sample was taken. In the case of the phyllosphere, the shoot samples were washed with tap water for 5 min and then with distilled water for 5 min. These tissues were dried at room temperature and a 1-g sample was macerated using sterilized pestle and mortar. Serial dilutions $(10^{-1}–10^{-10})$ were made for all samples (Somasegaran 1994). Dilutions from 10^{-4} to 10^{-6} were inoculated on Luria-Bertani agar (LB) plates for counting colony-forming units (CFU) per gram of dry weight. Plates were incubated at 37 °C until the appearance of bacterial colonies. Bacterial colonies were counted and the number of bacteria per gram sample was calculated. The bacteria were purified by repeated sub-culturing of single colonies. Single colonies of each isolate were selected, grown in LB broth, and stored in 30% glycerol at − 80 °C for subsequent characterization.

Identification of bacterial isolates based on 16S rRNA sequence

From individual bacterial strains, genomic DNA was extracted (Winnepenninckx et al. 1993). The 16S rRNA gene was amplified by using universal forward primer FD1 (AGAGTTTGATCCTGGCTCAG) and universal reverse primer (rP1) (ACGGACTTACCTTGTTACGACTT). A reaction mixture of 50 μL was prepared by using Taq buffer 5 μL (10×), MgCl2 5.5 μL (25mM), Taq polymerase 1.5 μL, dNTPs 4 μL (2.5mM), 4 μL of forward and reverse primer (10 pmol), and the template DNA 5 μL (> 50ng/μL) (Tan et al. 1997). Initial denaturation temperature was 95 °C for 5 min followed by 35 rounds of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min, and final extension at 72 °C for 10 min. These PCR products were purified and sequenced commercially by using the same universal forward and reverse primers (Eurofins, Germany).

Sequences of 16S rRNA were compared to those sequences deposited in the GenBank nucleotide database by using the NCBI BLAST. These sequences were aligned by using Clustal W software. A neighbor-joining tree was constructed by the bootstrap test with 1000 replicates (Saitou and Nei 1987). The evolutionary distances were compared using the maximum composite likelihood method in MEGA7

software (Tamura et al. 2004; Kumar et al. 2016). The 16S rRNA sequences of bacterial strains were deposited in the GenBank with accession numbers MT317180-MT317216.

Antibiotic resistance assay using the disk diffusion method

The antibiotic resistance pattern of bacterial strains was studied according to the Kirby-Bauer disk diffusion method (Bauer et al. 1966; El-Sayed and Helal 2016). Five antibiotics ampicillin (AMP), amoxicillin (AM), erythromycin (E), ciprofloxacin (CIP), tobramycin (TOB), gentamicin (GN), and vancomycin (VA) were used to check the antibiotic sensitivity of bacterial strains. Antibiotic disks were placed over freshly prepared LB medium seeded with bacterial strains under study. All antibiotic disks were placed on each of the seeded plates at appropriate distances from one another and plates were incubated at 37 °C for 48 h. The strains were classified as sensitive or susceptible if they showed a growth inhibition zone around the antibiotic disk.

Analysis of heavy metal resistance

A total of 18 bacterial strains from the rhizosphere and phyllosphere of *A. fatua* and 19 bacterial strains from the rhizosphere and phyllosphere of *B. reptans* were tested for resistance of cadmium (Cd), lead (Pb), and chromium (Cr). About 2, 5, 7.5, and 10 mM of each metal was used to analyze the resistance in the bacterial strains isolated using LB agar plates supplemented with these heavy metals. The concentration of heavy metals in the studied polluted soils was > 15 mg kg^{-1} and is considered not safe for plant growth.

Antimicrobial activity

On the basis of antibiotics and heavy metals resistance, 8 bacterial strains from the rhizosphere and phyllosphere of *A. fatua* and 10 bacterial strains from the rhizosphere and phyllosphere of *B. reptans* were tested for antimicrobial activity against six pathogenic bacterial strains. Antimicrobial activity test of bacterial strains isolated from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans* was performed by using the drop test method as described by Rao et al. (2005) with little modifications. Six pathogenic bacterial strains including *Bacillus cereus* (LT221128), *Staphylococcus aureus* (MT355444), *Pseudomonas aeruginosa* (LT797517), *Escherichia coli* (MT355445), *Klebsiella oxytoca* (LT221131), and *Enterobacter cloacae* (AM778415) were used for antimicrobial activity in this study. All the pathogenic strains were grown in 20 mL of LB broth at 37 °C for 24 h. The bacterial strains identified in this study were also grown in 50 mL of LB broth at 37 °C for 24 h. These cultures were centrifuged at 10,000 rpm for

15 min and the cell pellet was dissolved in 25 mL of saline water (1% NaCl). About 100 μL of a pathogenic strain was spread on LB agar plate and dried for 30 min. Then, a drop (10 μL) of the target bacterial culture with about 10^{10} cells mL^{-1} was spotted on this plate and incubated at 37 °C for 48 h. The zone of inhibition around the tested strain was measured in mm (millimeter).

Genome sequencing and annotation of *P. putida* BR-PH17

Pseudomonas putida BR-PH17 showed maximum potential for heavy metals and antimicrobial resistance. The next-generation whole-genome sequencing was performed by using Illumina Hiseq 2000 platform. Paired-end genome fragments were annealed to the flow-cell surface in a cluster station (Illumina). Sequencing-by-synthesis was performed with a total of 100 cycles. All reads were quality filtered and assembled using the A5 pipeline, an integrated pipeline for de novo assembly of microbial genomes (Tritt et al. 2012). The final genome coverage was 181× with a genome size of 5.78 Mbp. Genome annotation was performed by using NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al. 2016). The coding genes were predicted by using an ab initio gene prediction algorithm with homology-based methods. The gene function was annotated by BLAST against the Kyoto Encyclopedia of Genes and Genomes database KEGG pathway (Kanehisa et al. 2006). To predict genes and operons involved in secondary metabolism and antibiotic resistance, antiSMASH 4.0 software was used (Blin et al. 2017). The chromosomal DNA and plasmid sequence were deposited in the GenBank database under the accession number CP066306 and CP066307 (BioProject: PRJNA685985).

Statistical analysis

One-way ANOVA with Student's *t*-test was applied to analyze differences among the physicochemical characteristics of rhizospheric soils and significance at the 5% level was tested by least significance difference test (LSDT) by using STATISTIX software v. 8.2 (Steel et al. 1997).

Results

Physical and chemical characteristics of rhizospheric soils

The physical and chemical analysis showed that the rhizospheric soils of *A. fatua* were more alkaline and saline as compared to soils *B. reptans* (Table 1). There is no significant difference in soil moisture content and temperature in the rhizospheric soils of both plants. All the polluted soils had

Table 1 Physical and chemical properties of rhizospheric soils of *Avena fatua* and *Brachiaria reptans*

Soil properties	*Avena fatua*	*Brachiaria reptans*
pH	8.02[b]	7.26[a]
EC$_{1:1}$ (dS/m)	4.51[a]	3.73[b]
Moisture (%)	19.23[a]	21.61[a]
Temperature (°C)	31.31[b]	32.47[a]
Texture class	Silty loam	Silty loam
OM (g Kg^{-1})	17.51[b]	14.41[a]
NO^{3-} (mg kg^{-1})	12.83[a]	8.71[a]
Cd (mg kg^{-1})	35[a]	49[b]
Pb (mg kg^{-1})	43[b]	25[a]
Cr (mg kg^{-1})	21[b]	17[a]

Note: *EC*, electrical conductivity; *OM*, organic matter; *P*, phosphorous; *K*, potassium; *Ca*, calcium; *Mg*, magnesium; NO^{-3}, nitrate ion; values are mean of three samples (mean ± SE); letters represent statistically different values at 5% level

high concentrations of heavy metals Cd, Pb, and Zn (Table 1). The concentration of heavy metals in the studied polluted soils was > 15 mg kg^{-1} and is considered not safe according to Food Safety and Standards (Contaminants, Toxins, and Residues) Regulation Committee. The rhizospheric soils of *A. fatua* showed 35 mg kg^{-1} of Cd, 43 mg kg^{-1} of Pb, and 21 mg kg^{-1} of Cr respectively. The rhizospheric soils of *B. reptans* showed 49 mg kg^{-1} of Cd, 25 mg kg^{-1} of Pb, and 17 mg kg^{-1} of Cr respectively.

Identification of bacterial strains based on 16S rRNA analysis

A total of 8 bacterial strains from the rhizosphere and 10 bacterial strains from the phyllosphere of *A. fatua* were identified on the basis of 16S rRNA gene analysis. Three strains AV-RO1, AV-RO6, and AV-RO8 showed more than 99% similarity with *Bacillus* spp.; AV-RO2 strain had 99% homology with *Pseudomonas fluorescens* and bacterial strains belonging to *Virgibacillus* sp.; and *Enterococcus durans*, *Kocuria rosea*, and *Nocardia* were also identified from the rhizosphere of *A. fatua* (Table S1; Fig. 1A). While from the phyllosphere of *A. fatua*, four strains including AV-HP1, AV-HP2, AV-HP4, and AV-HP10 were identified as different species of *Bacillus*, AV-HP3 strain identified as *Staphylococcus equorum*, AV-HP5 strain as *Pseudomonas plecoglossicida*, AV-HP7 strain as *Enterococcus durans*, AV-HP8 as *Nocardia farcinica*, AV-HP6, and AV-HP11 as *Enterobacter aerogenes* (Table S1; Fig. 1A).

From the rhizosphere of *B. reptans*, 4 strains were related to *Bacillus* spp., 2 bacterial strains BR-RO1 and BR-RO10 were identified as *Staphylococcus equorum*, BR-RO4 strain was belonging to the *Marinobacter* sp., and BR-PH5 strains were
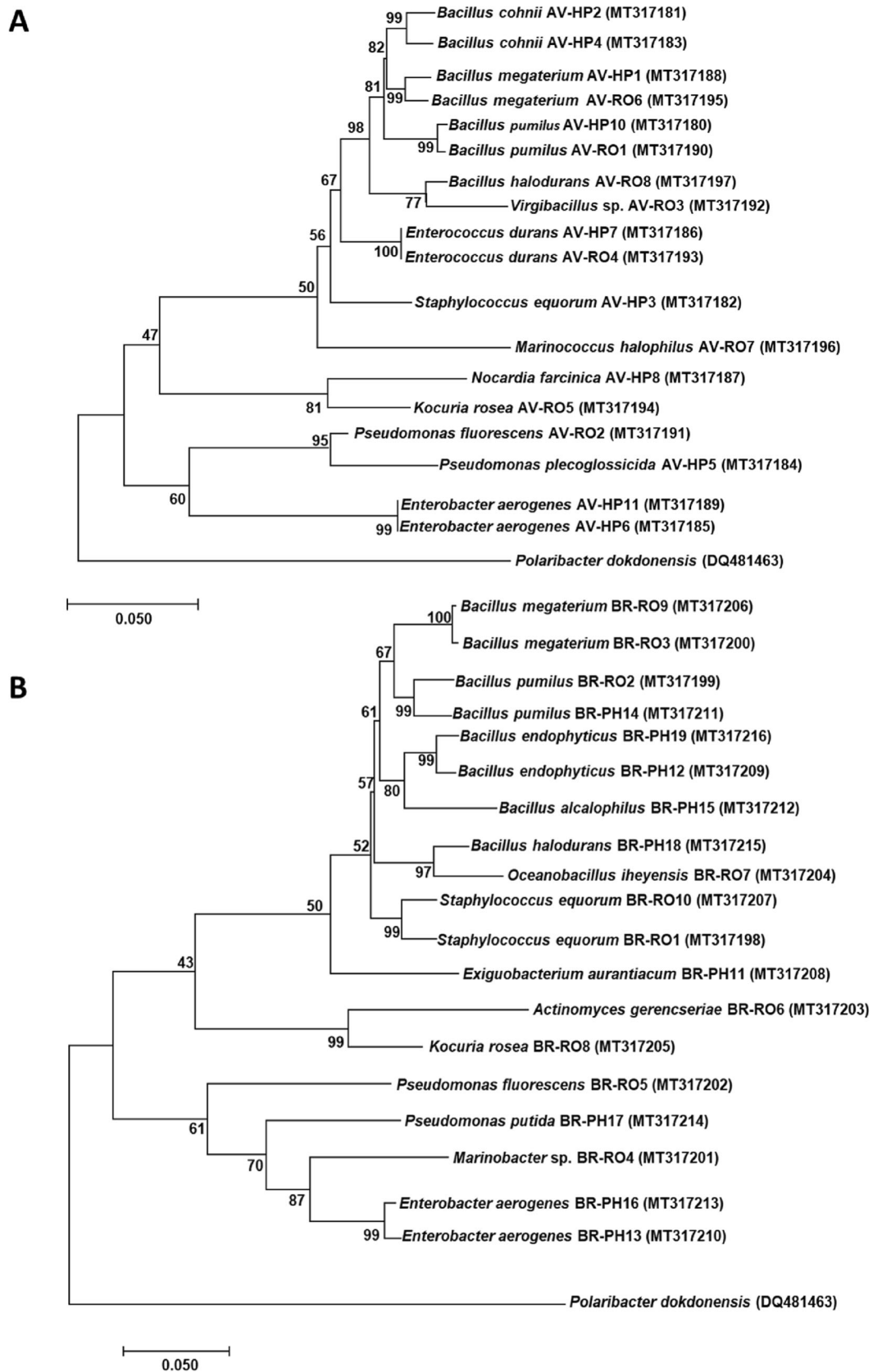
Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences of bacterial isolates associated with from the rhizosphere and phyllosphere of *A. fatua* (**A**) and *B. reptans* (**B**). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches

identified as *Pseudomonas fluorescens* (Table S2; Fig. 1B). From the phyllosphere of *B. reptans*, out of nine, 5 bacterial strains were identified as different species of *Bacillus*, BR-PH11 strain had more than 99% similarity with *Exiguobacterium aurantiacum*, BR-PH13 and BR-PH16 strains were identified as *Enterobacter aerogenes*, and BR-PH17 strain was identified as *P. putida* (Table S2; Fig. 1B). Overall, *Bacillus* and *Enterobacter* were the dominating bacterial genera in the rhizosphere and phyllosphere of both plants.

Antibiotic resistance profile of bacterial strains

About 50% bacterial strains showed resistance against both ampicillin and amoxicillin; 20% bacterial strains showed resistance against both erythromycin and tobramycin; 10% bacterial strains showed resistance against each ciprofloxacin, gentamicin, and vancomycin from the rhizosphere of *A. fatua*, while 55% bacterial strains showed resistance against both ampicillin, and amoxicillin; 37% bacterial strains showed resistance against ciprofloxacin; 25% bacterial strains showed resistance against each erythromycin, gentamicin, and vancomycin; and 12.5% strains were resistant to tobramycin from the phyllosphere of *A. fatua* (Table 2; Fig. S1).

From the rhizosphere of *B. reptans*, 30% bacterial strains showed resistance against both ampicillin and vancomycin, 50% bacterial strains showed resistance against both amoxicillin and erythromycin, 40% bacterial strains showed resistance against both ciprofloxacin and gentamicin, and 20% bacterial strains resisted against tobramycin, while from the phyllosphere of *B. reptans*, maximum bacterial strains (55%) showed resistance against amoxicillin; 33% bacterial strains showed resistance against each ampicillin, erythromycin, and tobramycin; 22% bacterial strains showed resistance against both ciprofloxacin and vancomycin; and 44% bacterial strains were resistant to gentamicin (Table 2; Fig. S1).

Heavy metal resistance profile of bacterial strain

More than 80% of the rhizospheric bacterial strains and 90% of the phyllospheric bacterial strains from both plants showed Cd tolerance at a concentration of 2 mM, 71–78% bacterial strains showed Cd tolerance at a concentration of 5 mM and only few strains (0–19%) from the rhizosphere, and 22–37% of bacterial strains from the phyllosphere of *A. fatua* and *B. reptans* showed Cd tolerance concentration of 10 mM (Table S3; Fig. 2A).

Similar results were obtained in case of Pb resistance. About 80–89% bacterial strains from the rhizosphere and 91–99% of bacterial strains from the phyllosphere of *A. fatua* and *B. reptans* showed Pb resistance at a concentration of 2 mM, 60% of bacterial strain from the rhizosphere, and 71% bacterial strains from the phyllosphere of both plants showed Pb resistance at a concentration of 5 mM and phyllospheric bacterial strains from both plants showed Pb

resistance (21–36%) as compared to rhizospheric bacterial strains with only 9–10 % Pb resistance (Table S4; Fig. 2B).

About 60–75% of rhizospheric bacterial strains and 77–87% of the phyllospheric bacterial strains from *A. fatua* and *B. reptans* showed Cr resistance at a concentration of 2 mM, 49–51% from the rhizosphere, and 57–64% of bacterial strains from the phyllosphere of both plants. None of the rhizospheric bacterial strains was able to tolerate 10 mM Cr concentration while 11–24% of the phyllospheric bacterial strains from both plants showed Cr tolerance at a concentration of 10 mM (Table S5; Fig. 2C).

Antimicrobial activity of bacterial strains

From the rhizosphere and phyllosphere of *A. fatua*, 62% strains had antimicrobial activity against *E. cloacae*, 50% strains showed antimicrobial activity against *P. aeruginosa*, 50% bacteria strains showed positive results against *E. coli*, 49% strains had antimicrobial activity against *K. oxytoca*, 20% strains showed antimicrobial activity against *B. cereus*, and 10% strains showed antimicrobial potential against *S. aureus* (Table 3; Fig. 3).

Similar results were obtained in the case of bacterial strains isolated from the rhizosphere and phyllosphere of *B. reptans*. About 70% of strains showed antimicrobial activity at least against three pathogenic strains. About 60% of bacterial strains showed antimicrobial potential against *K. oxytoca* and *P. aeruginosa* each, 50% of bacterial strains showed antimicrobial activity against *E. coli* and *E. cloacae*, and 30% of strains had antimicrobial activity against *B. cereus* and *S. aureus* (Table 3; Fig. 3).

General features of chromosomal and plasmid DNA of *P. putida* BR-PH17

The genome size of *P. putida* BR-PH17 is 5,774,421 bp with an average GC content of 61.07% (Fig. 4). A total of 5423 genes were identified and total coding sequences were 5321. The protein-coding CDSs were 5241 and 102 RNA genes with 22 rRNA genes, 75 tRNA genes, and 5 non-coding RNA genes were present on the chromosome (Table 4). A total of 80 genes were predicted as pseudo-genes because of missing C or N terminus or frameshift mutations.

The functional analysis of these genes using the KEGG pathway database showed that they have an important role in various metabolic pathways including plant growth promotion, bioremediation of different toxic compounds, heavy metal and antimicrobial resistance, and other abiotic stresses. Many small proteins detected were also annotated as hypothetical proteins. The functional analysis of CDSs showed that they could be classified into 22 general COG categories including the metabolism of carbohydrates, amino acids, lipids, transcription, energy, cofactors and vitamins, inorganic ions,

Table 2 Antibiotics resistance profile of bacterial strains isolated from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans* by the disk diffusion method

Plant zone	Bacterial isolates	Strain name	Antibiotics (diameter of clear zone in mm*)						
			AMP (30 μg)	AM (10 μg)	E (15 μg)	CIP (30 μg)	TOB (10 μg)	GN (10 μg)	VA (30 μg)
Rhizosphere of A. fatua	AV-RO1	*Bacillus pumilus*	+	++	++	++	++	−	++
	AV-RO2	*Pseudomonas fluorescens*	−	++	++	+	++	++	++
	AV-RO3	*Virgibacillus* sp.	+	+	−	++	++	−	−
	AV-RO4	*Enterococcus durans*	−	−	++	−	++	++	++
	AV-RO5	*Kocuria rosea*	++	−	++	+++	−	++	+
	AV-RO6	*Bacillus pumilus*	+	++	++	−	+++	−	+
	AV-RO7	*Marinococcus halophilus*	−	−	+	−	++	−	+
	AV-RO8	*Bacillus halodurans*	+	−	+++	++	++	++	++
Phyllosphere of A. fatua	AV-HP1	*Bacillus megaterium*	−	−	++	++	++	++	++
	AV-HP2	*Bacillus cohnii*	−	++	++	++	0.00	++	++
	AV-HP3	*Staphylococcus equorum*	−	++	++	−	+	+	++
	AV-HP4	*Bacillus cohnii*	−	−	++	++	++	−	+++
	AV-HP5	*Pseudomonas plecoglossicida*	+	+	−	+	−	+++	++
	AV-HP6	*Enterobacter aerogenes*	−	−	++	++	+++	+	+
	AV-HP7	*Enterococcus durans*	−	++	++	++	++	+++	++
	AV-HP8	*Nocardia farcinica*	++	−	+	++	++	++	++
	AV-HP10	*Bacillus megaterium*	−	−	++	++	++	++	-
	AV-HP11	*Enterobacter aerogenes*	−	−	++	++	+	++	++
Rhizosphere of B. reptans	BR-RO1	*Staphylococcus equorum*	−	−	++	−	++	−	−
	BR-RO2	*Bacillus pumilus*	++	++	++	++	+++	++	++
	BR-RO3	*Bacillus megaterium*	++	+++	+	−	++	−	+++
	BR-RO4	*Marinobacter* sp.	++	−	−	−	++	+	++
	BR-RO5	*Pseudomonas fluorescens*	+++	++	−	+	−	++	++
	BR-RO6	*Actinomyces gerencseriae*	+++	++	−	++	−	0.00	−
	BR-RO7	*Oceanobacillus iheyensis*	−	−	++	++	++	++	++
	BR-RO8	*Kocuria rosea*	+++	−	++	+	++	++	+
	BR-RO9	*Bacillus megaterium*	++	+++	+	+++	++	+++	++
	BR-RO10	*Staphylococcus equorum*	−	−	++	+++	++	−	−
Phyllosphere of B. reptans	BR-PH11	*Exiguobacterium aurantiacum*	++	−	++	++	−	−	++
	BR-PH12	*Bacillus endophyticus*	++	−	+++	++	−	++	++
	BR-PH13	*Enterobacter aerogenes*	++	+	++	+	++	−	++
	BR-PH14	*Bacillus pumilus*	+++	++	++	++	++	++	−
	BR-PH15	*Bacillus alcalophilus*	−	+	−	−	−	−	++
	BR-PH16	*Enterobacter aerogenes*	−	++	++	-	+	+	+
	BR-PH17	*Pseudomonas putida*	++	−	−	+++	++	+	++
	BR-PH18	*Bacillus halodurans*	+++	−	++	++	++	+++	++
	BR-PH19	*Bacillus endophyticus*	−	−	−	++	++	++	++

Note: *AMP*, ampicillin; *AM*, amoxicillin; *E*, erythromycin; *CIP*, ciprofloxacin; *TOB*, tobramycin; *GN*, gentamicin; *VA*, vancomycin; no inhibition zone (−) = resistant; a zone diameter < 16 mm (+) = weak susceptible; a zone diameter between 16 and 23 mm (++) = moderate susceptible; and a zone diameter with > 23mm (+++) = susceptible
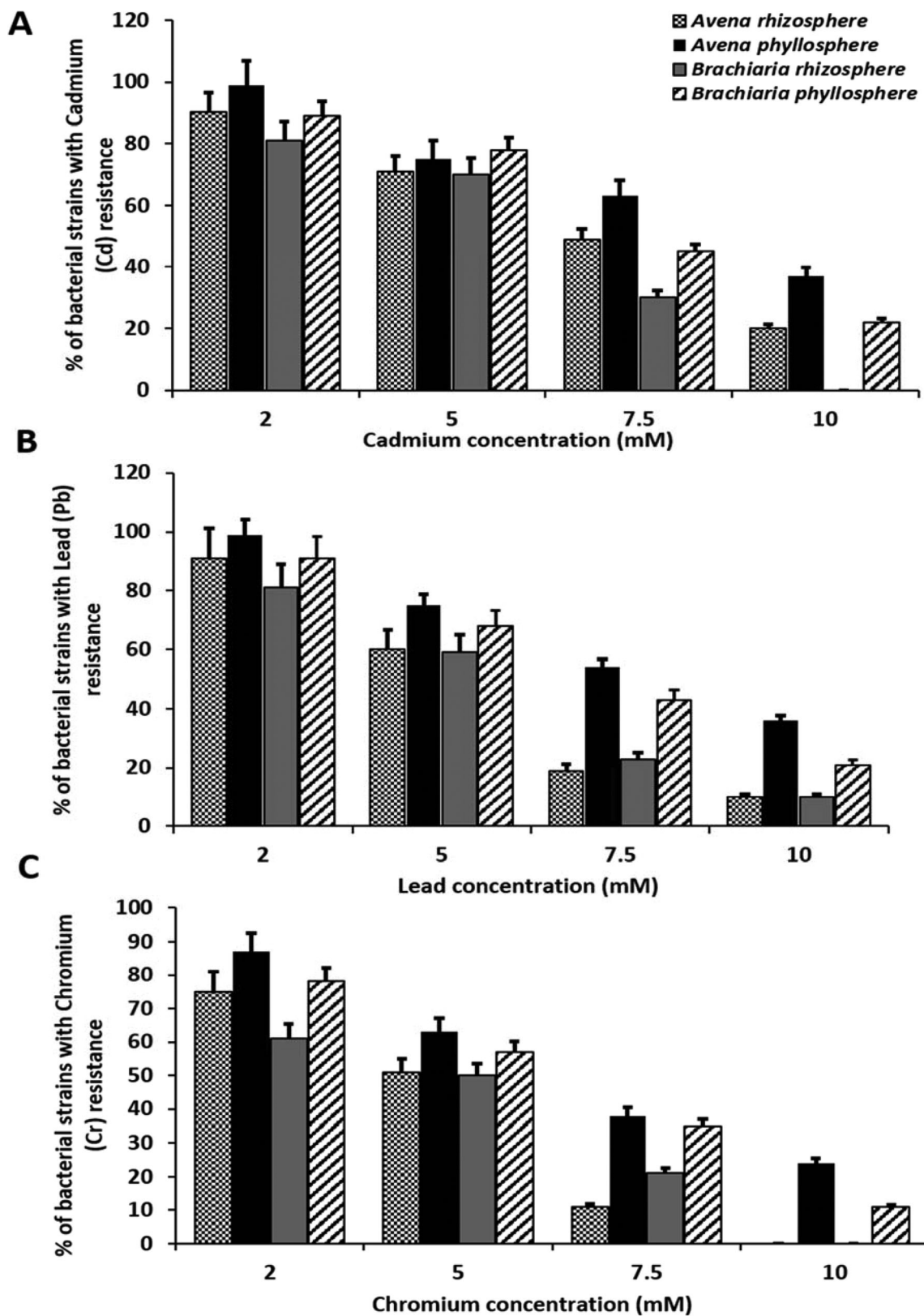
Fig. 2 Heavy metal resistance profile of bacterial strains isolated from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans*; **A** cadmium (Cd), **B** lead (Pb), and **C** chromium (Cr) with 2, 5, 7.5, and 10 mM concentrations

Table 3 Antimicrobial activity of bacterial strains isolated from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans*

Bacterial isolates	Antimicrobial activity (diameter of clear zone in mm*)					
	B. cereus	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. oxytoca*	*E. cloacae*
AV-RO1	0.00	0.00	25.31 ± 1.21	0.00	0.00	20.15 ± 2.12
AV-RO3	0.00	16.24 ± 1.29	0.00	0.00	23.58 ± 2.17	0.00
AV-RO7	14.65 ± 1.47	0.00	9.11 ± 1.77	15.22 ± 0.651	23.32 ± 1.99	10.37 ± 2.15
AV-RO8	0.00	0.00	0.00	0.00	0.00	15.41 ± 1.74
AV-HP2	0.00	0.00	14.35 ± 1.13	0.00	0.00	11.42 ± 2.01
AV-HP4	0.00	0.00	19.59 ± 2.95	12.31 ± 1.07	9.26 ± 1.07	0.00
AV-HP5	9.57 ± 0.28	0.00	0.00	21.41 ± 1.19	0.00	17.41 ± 1.87
AV-HP10	0.00	0.00	0.00	12.47 ± 1.17	20.17 ± 2.05	0.00
BR-RO3	0.00	15.63 ± 1.45	4.25 ± 0.52	0.00	21.69 ± 2.29	0.00
BR-RO4	21.57 ± 2.07	0.00	0.00	11.29 ± 2.06	19.17 ± 1.34	23.74 ± 1.53
BR-RO7	0.00	0.00	18.39 ± 1.45	0.00	16.58 ± 1.59	4.85 ± 0.61
BR-RO9	0.00	22.31 ± 2.37	0.00	20.14 ± 2.24	0.00	15.24± 1.56
BR-PH11	13.23 ± 1.27	0.00	15.21 ± 2.05	24.45 ± 2.75	0.00	0.00
BR-PH12	0.00	0.00	19.03 ± 1.58	0.00	0.00	18.67 ± 1.71
BR-PH15	0.00	0.00	0.00	0.00	6.57 ± 1.34	0.00
BR-PH17	15.31 ± 1.45	23.24 ± 2.39	13.39 ± 1.35	24.47 ± 1.87	0.00	17.21 ± 1.85
BR-PH18	0.00	0.00	17.34 ± 2.17	0.00	9.11 ± 1.59	0.00
BR-PH19	0.00	0.00	0.00	11.24 ± 1.28	18.93 ± 1.54	0.00

Note: Inhibition zones from ≥ 16 mm were considered a strong (+++), from 6 to 16 mm a moderate (++), and ≤ 6 mm a weak positive (+)

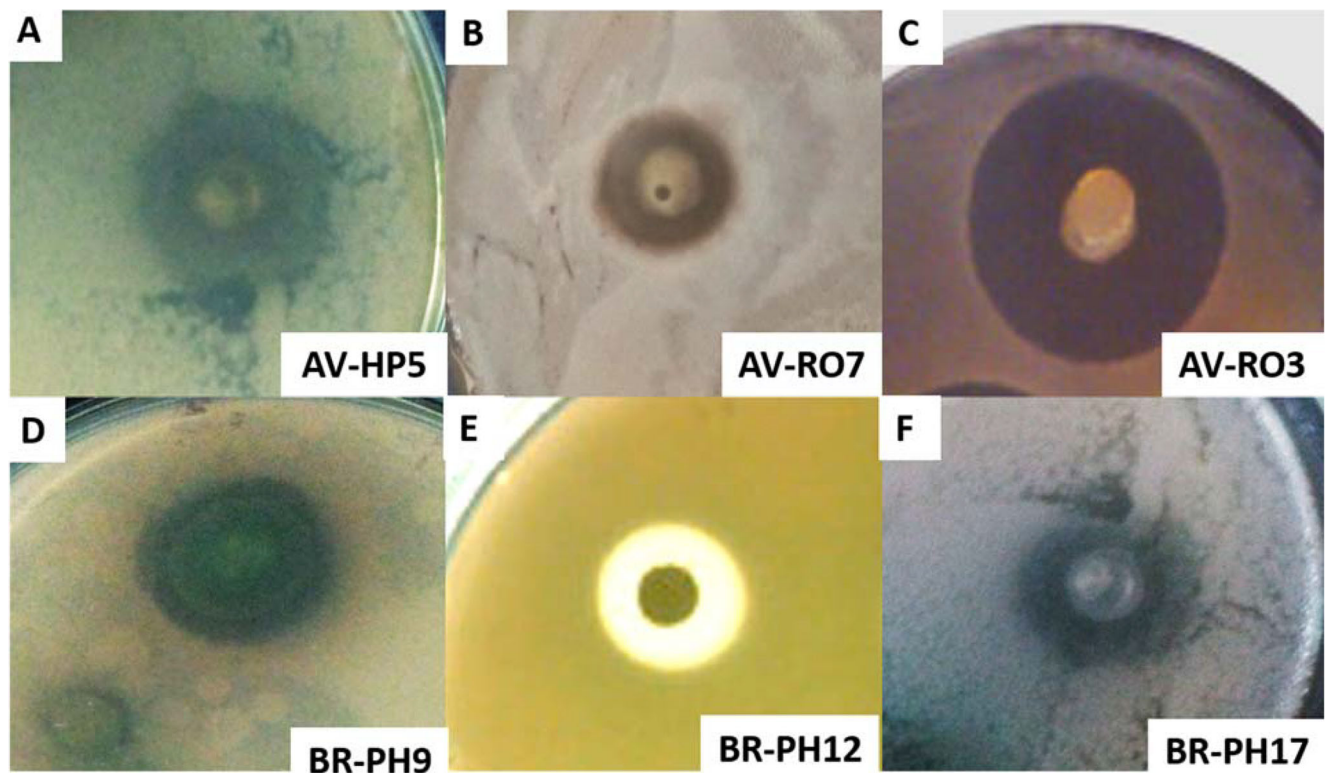*Average of three replicates ± standard error of means

Fig. 3 Antimicrobial activity of bacterial strains isolated from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans* using drop test method against pathogenic bacterial strains; **A** *B. cereus*, **B** *S. aureus*, **C** *P. aeruginosa*, **D** *E. coli*, **E** *K. oxytoca*, and **F** *E. cloacae*

Table 4 General features of *P. putida* BR-PH17 genome

Features	Value
Genome size (bp)	5,774,421
GC content	61.07%
Genes (total)	5423
CDS (total)	5321
Genes (coding)	5241
CDSs (with protein)	5241
Genes (RNA)	102
rRNAs	8, 7, 7 (5S, 16S, 23S)
Complete rRNAs	8, 7, 7 (5S, 16S, 23S)
tRNAs	75
ncRNAs	5
Pseudo-genes (total)	80

signal transduction and cellular processes, glycan biosynthesis and metabolism, cell motility, translation, ribosomal biogenesis, DNA replication and repair, secondary metabolites, defense mechanisms, and xenobiotics biodegradation (Table S6; Fig. 5A).

Whole-genome sequence analysis also showed that there was one plasmid pBR-PH17 with 80,360 bp size. A total of 97 genes were encoded by plasmid pBR-PH17. Functional analysis of these showed that 16% of genes involved in

xenobiotics biodegradation and metabolism, 14% genes coded different proteins and enzymes which caused human diseases, 11% genes involved in genetic information processing, 9% genes coded proteins and enzymes involved in energy metabolism, 7% genes involved in carbohydrate metabolism, 6% genes involved in amino acid metabolism, and 22% genes were unclassified (Table S7; Fig. 5B).

Prediction of antimicrobial-resistant proteins and enzymes from *P. putida* BR-PH17

Functional analysis showed that antimicrobial genes including tetracycline, phenicol, beta-lactam, cationic antimicrobial peptide (CAMP), vancomycin, aminoglycoside, sulfonamide, trimethoprim, rifampin, and multidrug drug resistance were encoded by chromosomal DNA while some antimicrobial genes are also encoded by plasmid DNA, e.g., tetracycline, aminoglycoside, sulfonamide, phenicol, and beta-lactam (Table S8; Fig. 6A and 6B).

Heavy metal resistance and bioremediation potential of *P. putida* BR-PH17

Based on functional genome analysis of *P. putida* BR-PH17, different heavy metal determinants were identified. Heavy metal resistance genes such as cadmium (Cd), lead (Pb),

Fig. 4 Circular genome of *P. putida* BR-PH17. From the outside in, circles 1 and 2 represent the coding regions with different COG categories, circle 3 represents mean-centered G+C content (bars facing outside-above mean, bars facing inside-below mean), and circle 4 shows GC skew (G2C)/(G+C). GC content and GC skew were calculated using a 10-kb window in steps of 300 bp
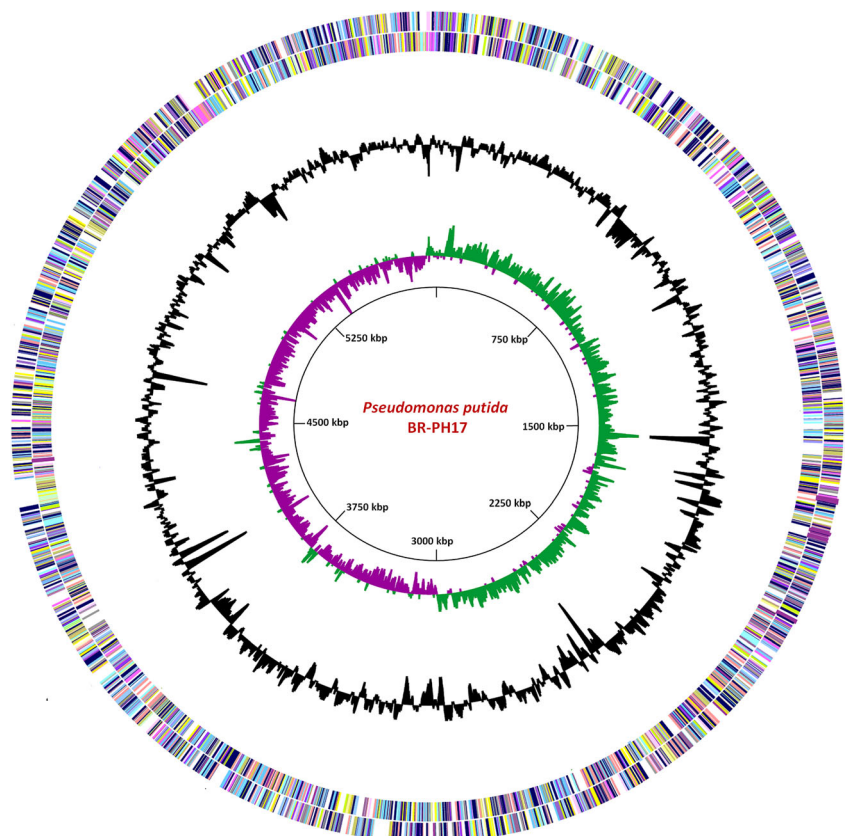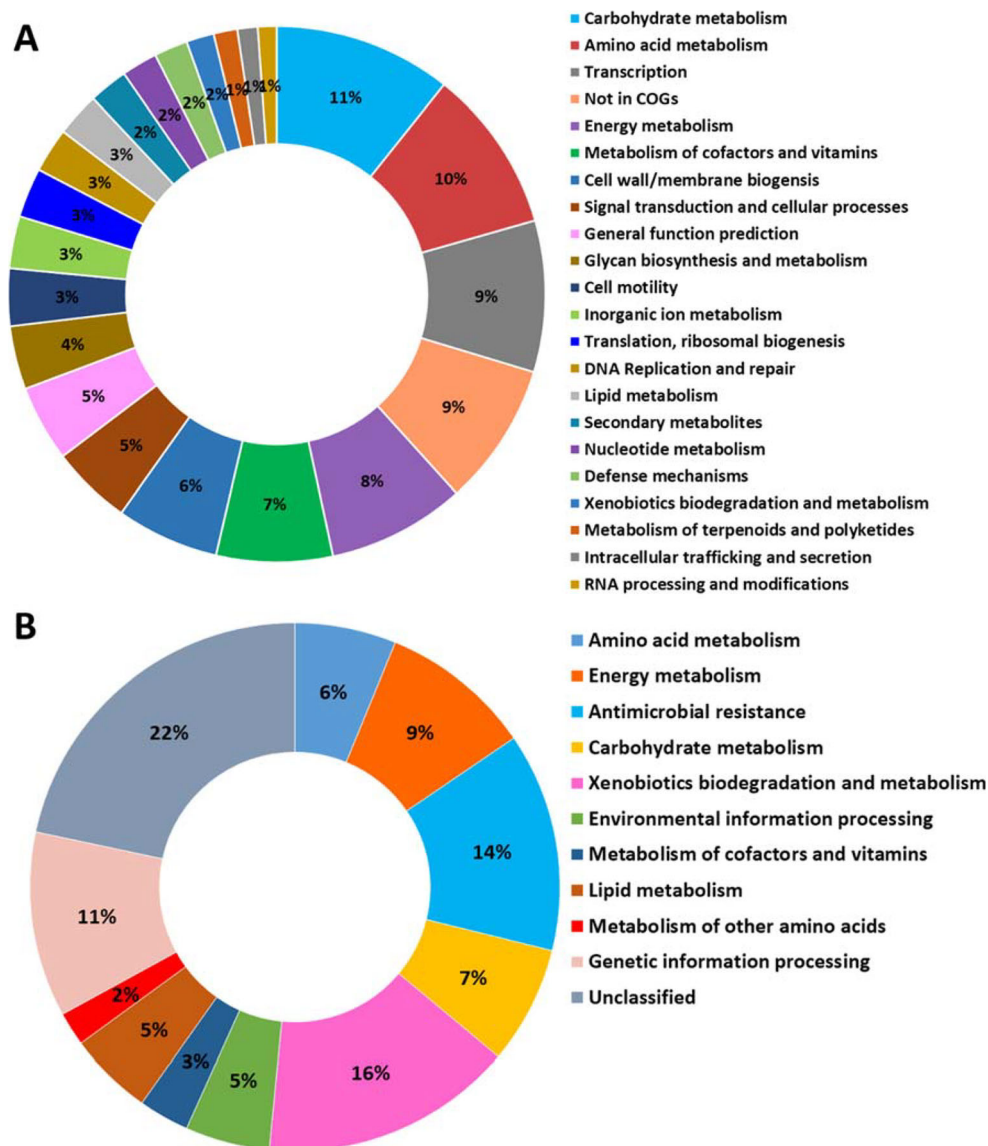
Fig. 5 Functional analysis of
P. putida BR-PH17 chromosomal
(**A**) and plasmid pBR-PH17 (**B**)
encoded genes by using KEGG
metabolic pathways

chromium (Cr), zinc (Zn), copper (Cu), nickel (Ni), and mercury (Hg) were encoded by chromosomal DNA and lead, cadmium, copper, zinc, cobalt (Co), and manganese (Mn) resistance genes were encoded by plasmid DNA (Table S9; Fig. 6C and 6D).

Identification of gene clusters involved in secondary metabolism of *P. putida* BR-PH1

Genome annotation of *P. putida* BR-PH1 showed that a number of gene clusters including NAGGN (N-acetylglutaminylglutamine amide), RiPPs (ribosomally synthesized and post-translationally modified peptides), ranthipeptide, phenazine, NRPS (non-ribosomal peptide synthetase) biosynthesis, and redox-cofactors involved in secondary metabolism were identified in the genome of *P. putida*

BR-PH1 which might be involved in plant growth improvement and biocontrol mechanisms (Fig. 7).

Discussion

In recent years, microbial diversity analysis from polluted environments is getting more attention due to the shortage of arable lands. Microbial communities associated with the plants growing under heavy metal pollution have great biotechnological potential that can be utilized for the bioremediation and restoration of these contaminated lands (Doni et al. 2012; Mazinani et al. 2017; Sun et al. 2017). The current study was the report of microbial diversity associated with the rhizosphere and phyllosphere of *A. fatua* and *B. reptans* growing in polluted areas near Lahore. Here, we also characterized
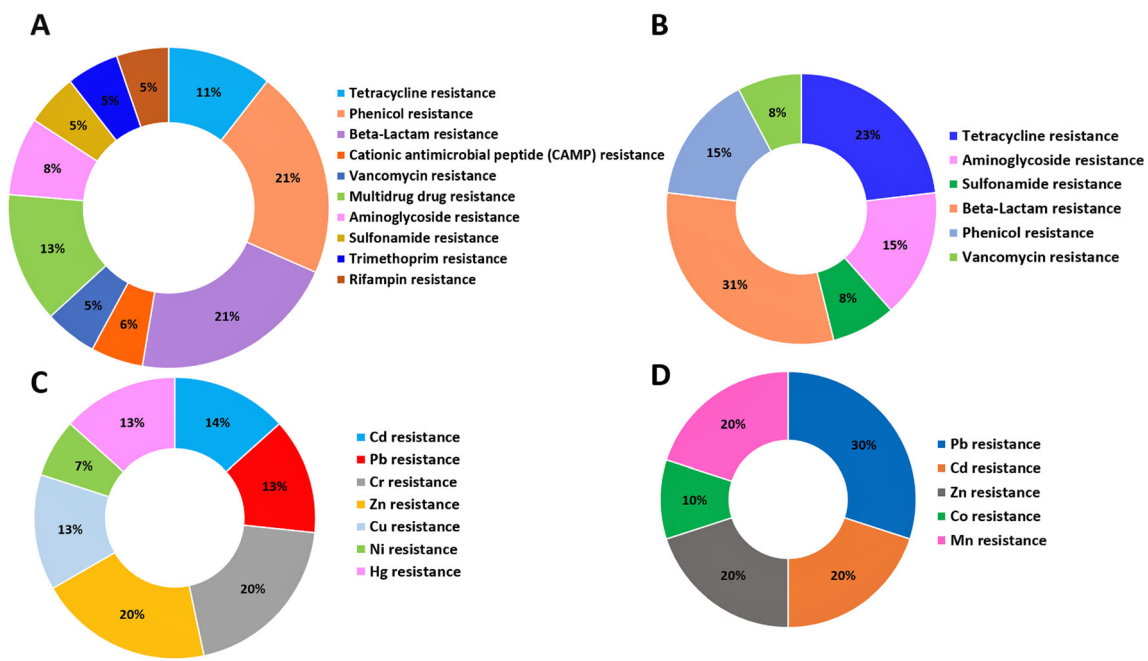
Fig. 6 Prediction of antimicrobial (**A** and **B**) and heavy metal resistance genes (**C** and **D**) in the genome of *P. putida* BR-PH17 through KEGG metabolic pathways

these strains on the basis of their antimicrobial and heavy metal resistance potential.

Bacterial genera from the phylum Firmicutes such as *Bacillus*, *Virgibacillus*, *Marinococcus*, *Staphylococcus*, *Exiguobacterium*, and *Enterococcus* were commonly identified from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans.* Members of Firmicutes have more potential to survive under polluted environments as compared to other bacterial phyla. A number of previous studies have already reported that *Bacillus*, *Staphylococcus*, and *Enterococcus* were dominant bacterial genera identified from the root and shoot endosphere of various plant species including grasses, citrus plants, and gymnosperms (Mwajita et al. 2013; Smith et al. 2015). *Bacilli* have a wide range of applications in agriculture, medicine, bioremediation of hazardous compounds, and various industries (Kumar et al. 2011; Mukhtar et al. 2018, 2019a).

Bacterial genera such as *Pseudomonas*, *Marinobacter*, and *Enterobacter* belonging to the phylum Proteobacteria also showed high abundance in the rhizosphere and phyllosphere of both plants. These bacterial strains have been identified from the rhizosphere and phyllosphere of several plants growing in contaminated or other stress-affected lands (Bodenhausen et al. 2013; Haroun et al. 2015). Bacterial strains related to *Kocuria* and *Nocardia* (Actinobacteria) were also identified and characterized in this study. These strains have great potential for plant growth promotion and

biocontrol of different plant diseases. Members of Actinobacteria produce a variety of antifungal and antibacterial compounds and play an important role in plant health and productivity (Miransari, 2013; Buedenbender et al. 2017; Zhao et al. 2018).

From both plants, about 40% of isolates showed resistance against ampicillin and amoxicillin. More than 65% of bacterial strains identified in this study were sensitive to erythromycin, ciprofloxacin, and gentamicin. A number of previous studies also reported that microbial diversity from polluted environments such as contaminated soil, water, and wastewater-activated sludge samples showed a variety of antibiotics resistance bacterial strains (Balcom et al. 2016; Meyer et al. 2016). When a genetic change mediates resistance to antibiotics and heavy metals or when antibiotic resistance and metal resistance genes are genetically linked on mobile genetic elements (co-resistance), metal can be used to co-select the clinically important antibiotics (Seiler and Berendonk 2012; Dickinson et al. 2019).

Bacterial strains from the rhizosphere of both plants showed overall better results for heavy metal resistance as compared to bacterial strains from the phyllosphere. More than 60% of bacterial strains from the rhizosphere and more than 50% of strains from the phyllosphere of both plants were able to tolerate up to 5 mM while only a few strains were able to grow at l0 mM of Cd, Pb, and Cr. Most bacterial strains isolated from polluted environments have antibiotics and
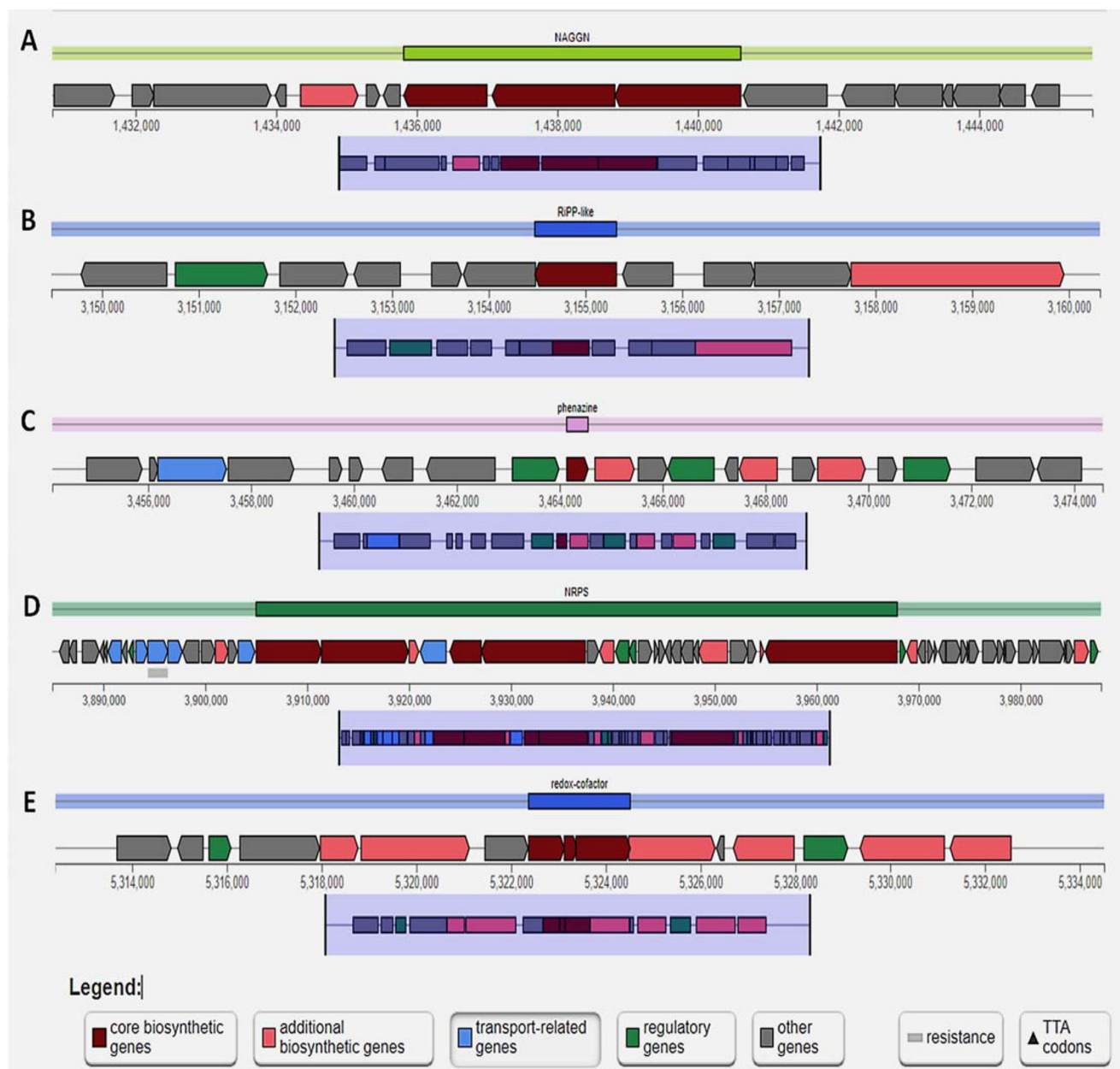
Fig. 7 Identification of gene cluster involved in secondary metabolism in the genome of *P. putida* BR-PH17 by using antiSMASH software

heavy metal resistance genes on their plasmids. These bacteria play a crucial role in the survival of host plants growing in contaminated lands (Qin et al. 2011; Schuffler and Kübler 2016). Bacterial diversity isolated from heavy metal-contaminated sites has the ability to spread antimicrobial resistance in those environments. These bacteria have evolved different physiological and genetic mechanisms to mediate metal stress (Francois et al. 2012; Dickinson et al. 2019).

From the rhizosphere and phyllosphere of *A. fatua*, *Bacillus*, *Pseudomonas*, and *Marinococcus* strains showed maximum antimicrobial activity against *P. aeruginosa*, *E. cloacae*, and *K. oxytoca* while the rhizosphere and phyllosphere of *B. reptans*, *Bacillus*, and *Pseudomonas* strains

showed maximum antimicrobial potential against *K. oxytoca*. *Bacillus*, *Pseudomonas*, *Vibrio*, and *Enterobacter* are well-known bacterial genera that have been studied as plant probiotic bacteria (Andreote et al. 2010; Wang et al. 2018; Chen et al. 2019). Okezie et al. (2020) reported the antibacterial activity of *Bacillus* sp. strain FAS against *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumonia* with zones of inhibition ranging from 15 to 28 mm. Some *Bacillus* species have been characterized for their production of antibacterial and antifungal compounds and their role in the protection of plants against different diseases (Gupta et al. 2015; Hung et al. 2007; Ismail et al. 2016; Mukhtar et al. 2019b). Some previous studies also reported that members

of Proteobacteria such as *Pseudomonas*, *Vibrio*, *Burkholderia*, *Klebsiella*, and *Enterobacter* showed antimicrobial activity against different pathogenic bacterial and fungal strains (Mazinani et al. 2017; Wang et al. 2018; Zhao et al. 2018).

The functional analysis of the *P. putida* BR-PH17 genome showed that 5241 protein-coding sequences were predicted with a large number of small proteins annotated as hypothetical proteins. The genes involved in the metabolism of carbohydrates, amino acids, lipids, transcription, energy, cofactors and vitamins, inorganic ions, glycan biosynthesis and metabolism, DNA replication and repair, signal transduction and cellular processes, cell motility, translation, ribosomal biogenesis, secondary metabolites, defense mechanisms, and xenobiotic biodegradation were mainly identified through KEGG pathways analysis. These proteins and enzymes were previously reported in genome analysis of a number of *P. putida* strains (Molina et al. 2014; Chong et al. 2016; Rodríguez-Rojas et al. 2016).

A distinctive characteristic of *P. putida* BR-PH17 was its resistance against various antibiotics and heavy metals as compared to other *Pseudomonas* strains characterized in this study. The results showed that it was highly resistant against tetracycline, phenicol, beta-lactam, cationic antimicrobial peptide (CAMP), vancomycin, aminoglycoside, sulfonamide, trimethoprim, rifampin, and multidrug drugs. A number of previous studies showed that most of the *Pseudomonas* strains showed resistance to different antibiotics, such as streptomycin, penicillin, tetracycline, kanamycin, vancomycin, erythromycin, and chloramphenicol (Irawati and Yuwono 2016; Mukhtar et al. 2019b; Wang et al. 2018). Analysis of *P. putida* BR-PH17 genome revealed the presence of various metal resistance genes such as Cd, Pb, Cr, Zn, Cu, Ni, Hg, Mn, and Co. Several studies have previously reported the different mechanisms for heavy metal tolerance in *Pseudomonads* (Chong et al. 2016; Mukhtar et al. 2019b; Schuffler and Kübler 2016). Several gene clusters involved in secondary metabolism, e.g., NAGGN, RiPPs, ranthipeptide, phenazine, NRPS biosynthesis, and redox-cofactors were also identified in the genome of *P. putida* BR-PH17. These gene clusters have also been reported by some previous studies on the genome sequence analysis of *Pseudomonas* strains isolated from different polluted environments (Kang et al. 2020; Singh et al. 2021).

Conclusion

To the best of our knowledge, the present study is the first report about the phylogenetic analysis of bacteria from the rhizosphere and phyllosphere of *A. fatua* and *B. reptans* collected from polluted sites of the Kala-Shah Kaku industrial area near Lahore. *Bacillus*, *Staphylococcus*, *Pseudomonas*,

and *Enterobacter* were the dominating bacterial genera identified from the rhizosphere and phyllosphere of *A. fatua* and *Bacillus*, *Marinobacter*, *Pseudomonas*, *Enterobacter*, and *Kocuria* were the dominating bacterial genera identified from the rhizosphere and phyllosphere of *B. reptans*. The bacterial strains identified in this study also showed great potential for heavy metal resistance and antimicrobial activity against different pathogenic bacterial strains. From the results of genome sequence analysis, it was predicted that *P. putida* BR-PH17 had various antimicrobial resistance genes such as tetracycline, beta-lactam, cationic antimicrobial peptide (CAMP), aminoglycoside, vancomycin, sulfonamide, and rifampin, and metal resistance genes such as Cd, Pb, Cr, Zn, Cu, Ni, Hg, Mn, and Co. By complete genome analysis of *P. putida* BR-PH17, we are able to explain biological interactions among different biomolecules and other microorganisms. The availability of the whole genetic contents of this organism will surely help to provide more insight in unraveling the complex biological mechanisms that *P. putida* BR-PH17 uses to develop antimicrobial and heavy metal resistance. Bacterial strains characterized from the rhizosphere and phyllosphere of plants growing in polluted areas can be utilized as a new source of antibiotics that might be used as promising antifungal and antibacterial drugs against different diseases.

Declarations

Ethical approval and consent to participate   Not applicable.

Consent for publication   Not applicable.

Conflicts of interest   The authors declare no competing interests.

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