Genetic diversity and characterization of arsenic-resistant endophytic bacteria isolated from Pteris vittata, an arsenic hyperaccumulator

Yunfu Gu*, Yingyan Wang†, Yihao Sun†, Ke Zhao, Quanju Xiang, Xiumei Yu, Xiaoping Zhang and Qiang Chen

Abstract

Background: Alleviating arsenic (As) contamination is a high-priority environmental issue. Hyperaccumulator plants may harbor endophytic bacteria able to detoxify As. Therefore, we investigated the distribution, diversity, As (III) resistance levels, and resistance-related functional genes of arsenite-resistant bacterial endophytes in Pteris vittata L. growing in a lead-zinc mining area with different As contamination levels.

Results: A total of 116 arsenite-resistant bacteria were isolated from roots of P. vittata with different As concentrations. Based on the 16S rRNA gene sequence analysis of representative isolates, the isolates belonged to Proteobacteria, Actinobacteria, and Firmicutes. Major genera found were Agrobacterium, Stenotrophomonas, Pseudomonas, Rhodococcus, and Bacillus. The most highly arsenite-resistant bacteria (minimum inhibitory concentration > 45 mM) were isolated from P. vittata with high As concentrations and belonged to the genera Agrobacterium and Bacillus. The strains with high As tolerance also showed high levels of indole-3-acetic acid (IAA) production and carried arsB/ACR3(2) genes. The arsB and ACR3(2) were most likely horizontally transferred among the strains.

Conclusion: The results of this study suggest that P. vittata plants with high As concentrations may select diverse arsenite-resistant bacteria; this diversity might, at least partly, be a result of horizontal gene transfer. These diverse endophytic bacteria are potential candidates to enhance phytoremediation techniques.

Keywords: Pteris vittata, Endophytic bacteria, Genetic diversity, Arsenite resistance, Arsenic transport genes, Horizontal gene transfer

Background

Arsenic (As) is a trace metalloid element present in various soil and water ecosystems and originated from both natural processes and human activities [1]. Chronic exposure to soil As possess potential health risks to environment and human health because of its toxicity and carcinogenicity [2]. Moreover, soil contamination with As affects the physiology, growth, and grain quality of crops. For example, the As concentrations in rice grains from Chenzhou, China, exceeded the maximum allowable value of 0.5 mg kg⁻¹ (dry weight) [3]. However, the efficient remediation of As-contaminated soil and water is a major environmental challenge, calling for the development of sophisticated remediation strategies.

Phytoremediation employs plants to accumulate, transfer, stabilize, and remove trace elements from polluted soils and water systems. It has received considerable attention due to its economic and environmental benefits [4]. However, there are several limiting factors affecting the effectiveness of phytoremediation, including plant growth rate, contaminant phytotoxicity, root biomass decrease, and limited uptake of contaminants [5].

In terms of suitable plant species, the arsenic hyperaccumulator plant P. vittata L. (Chinese brake fern) has
potential to be used in the phytoremediation of As-contaminated soils, since it is able to accumulate more than 1000 mg kg\(^{-1}\) of As in its fronds [6]. Plant-associated microbes could affect the efficiency of metal extraction by plants and thereby enhance phytoremediation processes [7, 8]. They play an important role in the transformation of arsenic, including arsenite (As(III)) oxidation, arsenate (As(V)) respiration, and As(V) reduction, thus affecting the bioavailability and toxicity of As in soils [9]. However, our knowledge about endophytic microorganisms associated with \textit{P. vittata} and their roles in As tolerance and transformation is severely limited.

Endophytic bacteria, which have great potential in enhancing phytoremediation processes [7, 10], can colonize internal plant parts, without any detrimental effects on the hosts [11]. Such bacteria are diverse and influenced by edaphic properties and contaminant contents of both the soil and plant tissue [12, 13]. Some endophytes can promote plant growth by improving nutrient uptake and increasing resistance to metals, suggesting their application in phytoremediation [14]. Bacteria have developed different strategies to transform arsenic, including cytoplasmic arsenate reduction, arsenite oxidation, respiratory arsenate reduction, and arsenite methylation [15]. The reduction of arsenate, followed by the extrusion (efflux) of arsenite, is the main mechanism of arsenate tolerance in bacteria [16]. Arsenite efflux is carried out by membrane carrier proteins or pumps, such as ArsB or ArsAB complexes and Acr3p. Although the arsenite transporter genes \textit{arsB} and \textit{Acr3p} have been identified in various soil bacterial species [17], knowledge about these genes in endophytic bacteria is still lacking. Moreover, the relation between genotypes and arsenite resistance levels has not been addressed. Thus, studying the diversity and distribution of indigenous bacterial endophytes in \textit{P. vittata} is crucial to improve phytoremediation strategies for As-contaminated sites.

In this context, we isolated endophytic bacteria from \textit{P. vittata} from a Pb-Zn mine soil for analysis of the 16S rRNA gene and arsenite transporter gene (e.g. \textit{arsB} and \textit{Acr3p}) diversity. The objectives of this study were to (1) evaluate the distribution and diversity of arsenite-resistant endophytic bacteria in \textit{P. vittata} with different arsenic contamination levels; and (2) investigate the arsenite transporter genes and correlate their presence to the arsenic resistance level of the bacteria. We hypothesized that the distribution and diversity of endophytic bacteria and their arsenite transporter genes are related to the As concentration in \textit{P. vittata} roots.

**Methods**

**Ethics statement**

No specific permits were required for the described studies. No specific permissions were required for these locations/activities because sample collection did not involve endangered or protected species or privately owned location.

**Study sites and sampling strategy**

Sampling was performed in the Tangjia Pb-Zn mine in Hanyuan, Sichuan (29°24’39’’ N, 102°39’24” E), 890 m above sea level. The area has a mean annual temperature of 18.5 °C, with a mean annual precipitation of 486 mm and an average annual evaporation of 1553 mm. Based on the findings of a previous field survey, \textit{P. vittata} is the dominant species in this area [18].

With regard to the distribution of the slag heaps, four different soils within the mining area, with different arsenic contamination levels, were selected: an ore outlet (site S2) and a tailing dam with smelting wastes and wastewater (site S3) with high As-contamination levels, and an ore charge heap (site S4) and a slag heap (site S5) with intermediate levels. A field used to cultivate summer rice and winter wheat, approximately 10 km from the mine area, was chosen as a non-contaminated control site (site S1). Soil and ferns samples (reproductive stage) were collected in August 2016 with the permission of the land owner. For this, three plots of 16 m × 10 m were randomly established in each site; each plot was divided into four 8 m × 5 m sampling subplots. Fresh roots and the corresponding rhizosphere soils from three individual \textit{P. vittata} plants were sampled from each subplot. The samples from the subplots were pooled and homogenized to form a composite plot-level sample.

Root and soil samples were placed in polyethylene bags at 4 °C and transported to the laboratory for analyses. For metal analysis, roots were washed with distilled water and dried to constant weight at 55 °C. Dried roots were ground into a fine powder and digested with HNO\(_3\)/HClO\(_4\) (87/13 v/v). Soil samples were air-dried at ambient room temperature (25 °C) and sieved through a 6-mm sieve. Then metals in the soil samples were extracted with \textit{aqua regia}. Total concentrations of As, Pb, Zn, Cu, and Cd in root extractant and \textit{aqua regia} were determined using an inductively coupled-plasma optical emission spectrometer (ICP-AES, IRIS Intrepid II, Thermo Electron, USA) [19]. Soil pH was determined with a potentiometry method, using a soil-to-water ratio of 1:5 [20]. Soil water content (WC) was determined after oven-drying at 105 °C for 48 h [20]. Soil total nitrogen (TN) and organic carbon (SOC) were analyzed via the potassium dichromate oxidation-external heating method and the alkaline hydrolysis diffusion method, respectively [20].

**Isolation of as-resistant endophytic bacterial strains**

The \textit{P. vittata} root samples were washed with tap water, followed by three rinses with deionized water and
sterilization by sequential immersion in 70% (v/v) etha-

nol for 2 min and 1% mercuric chloride for 1 min; sub-
sequently, samples were rinsed three times with sterile
water. Water from the last rinse was plated on Luria–
Bertani’s (LB) agar to test whether the root surface was
successfully disinfected. Surface-sterilized roots (ap-
proximately 0.5 g) were ground by a mortar and pestle
in 5 ml of ddH2O. Sterile quartz sand was added to the
mixture to improve cell wall disruption. Serial dilutions
were plated on LB medium containing 800 µM NaAsO2
and incubated at 28 °C for 7 days. Single colonies were
picked and restreaked several times to obtain pure iso-
lates. Isolates were stored on LB medium at 4 °C.

Genomic DNA extraction, PCR amplification, and ARDRA
Genomic DNA of the isolates was extracted from iso-
lates grown in 5 mL of LB liquid medium at 28 °C with
150 rpm for 18 h, using the standard phenol-chloroform
method described by Chang et al. [21]. The 16S rRNA
gene was amplified in a T100™ thermal cycler (Bio-Rad,
Hemel Hempstead, UK), using a pre-denaturation step
at 94 °C for 4 min, followed by 30 cycles of 50 s at 94 °
C, 55 s of annealing at 57 °C, 80 s extension at 72 °C,
and a final extension for 8 min at 72 °C. The PCR mix-
ture (25 µL) contained 50 ng of DNA template, 1.5 mM
MgCl2, 2.5 U Taq DNA polymerase (Invitrogen, USA),
1× PCR buffer, 100 pmol of the primers BSF 8/20: 5′-
AGAGTTTGATCCTGGCTCAG-3′, BSR1541/20: 5′-
AAGGAGGTGATCCAGCCGCA-3′ [22], and 200 µM
of each dNTP. The PCR products were checked by elec-
trophoresis in a 1% (w/v) agarose gel with 0.5
μg mL−1 ethidium bromide; subsequently, obtained PCR products
(1.5 kb) were purified with a Gel Extraction Kit (SBS
Genetech, Shanghai, China).

In the amplified rDNA restriction fragment analysis
(ARDRA), purified PCR products (about 5 µl) were
digested at 37 °C for 4 h, using the restriction enzymes
HhaI, HaeIII, MspI, and TaqI. Based on colony morph-
ology and the 16S rRNA gene ARDRA pattern, represen-
tative isolates were selected for 16S rRNA gene
sequencing in an ABI3730XL automatic sequencer at
Sangon Biotech (Shanghai, China).

Amplification of arsB and ACR3(2) genes
The arsB genes were amplified with the degenerate
primers dasrB1F (5′-GGTGTGGAACATC-GTCTGGA
AYGCNAC-3′) and dasrB1R (5′-CAGGCGTACAC-
CACCGTACATNC3-3′) [17]. The ACR3 (2) genes
were amplified with the primers dracr5F (5′-TGAT
CTGGGTCTGATCTTCCC- VATGMGTGVT-3′) and
dacr4R (5′-CGGCCAGGCCAGTYCTRAAARTT-3′)
[17]. The PCR products were purified with a Gel Extrak-
tion Kit (SBS Genetech, Shanghai, China) and sequenced
as described above.

DNA sequencing and phylogenetic analysis
All amplified products were sequenced using an
ABI3730XL automatic sequencer at Sangon Biotech
(Shanghai, China). Similar sequences were searched
using BlastN for 16S rRNA gene and BlastX for arsB/
Acr3p. Sequences from the isolates and reference se-
quences from GenBank were checked manually and edi-
ted to the same lengths, using the software package
ClustalX 2.0 [23]. Phylogenetic trees were constructed
by the neighbor-joining distance method, using the soft-
ware package MEGA 5.0 [24]. The reliability of inferred
trees was tested with 1000 bootstrap replicates.

Determination of arsenite resistance and indole-3-acetic
acid (IAA) and siderophore production
The minimum inhibitory concentrations (MICs), defined
as the lowest concentrations of arsenic as NaAsO2 that
inhibited growth in chemically defined medium (CDM)
broth, were tested in triplicate. Stock solutions of
NaAsO2 were prepared in ddH2O and sterilized. Isolates
were inoculated in 3 mL of CDM broth supplemented
with 6, 8, 10, 15, 20, 25, 30, 36, 42, and 48 mM NaAsO2
and incubated at 28 °C for 4 days. Low salt phosphate
(LSP) agar plates without NaAsO2 were used as controls.
The IAA production was measured as described by
Sheng et al. [25], while production of siderophores was
evaluated using the chrome azurol-S (CAS) analytical
method [26, 27].

Nucleotide sequence accession numbers
The sequences obtained in this study were deposited in
the NCBI GenBank database under accession numbers
MF185755–MF185785 for 16S rRNA genes, MF185786–
MF185799 for arsB, and MF185799–MF185803 for
ACR3(2).

Statistical analysis
Analysis of variance and the Student–Newman–Keuls
(SNK) test (P < 0.05) were used to compare treatment
means. All analyses were performed using SPSS 13.0 for
Windows (SPSS Inc., Chicago, USA).

Results
Soil physicochemical parameters and metal contents
All soils in the sampling sites were alkaline. However,
the pH in the mine area was significantly higher than
that in the non-contaminated control site S1 (Table 1).
Soil WC in the control site was higher than in the min-
ing area. The highest TN and SOC levels were detected
at S1 and the lowest at S5. The highest concentrations
of Cu, Cd, Zn, Pb, and As were detected at S3 (Table 1).
Table 1: The physico-chemical properties and heavy metal contents of the soils

| Sampling site | pH   | WC (%) | TN (g kg⁻¹) | SOC (%) | Cu (mg kg⁻¹) | Cd (mg kg⁻¹) | Zn (mg kg⁻¹) | Pb (mg kg⁻¹) | As (mg kg⁻¹) |
|---------------|------|--------|-------------|---------|--------------|--------------|--------------|--------------|--------------|
| S1            | 7.55 ± 0.07c | 6.26 ± 0.43a | 0.25 ± 0.03a | 4.76 ± 0.26a | 0.12 ± 0.01c | 0.07 ± 0.01e | 53.4 ± 3.35d | 15.6 ± 1.12e | 5.36 ± 0.87c |
| S2            | 7.78 ± 0.02a | 2.32 ± 0.62c | 0.12 ± 0.02b | 4.37 ± 0.08a | 0.88 ± 0.05b | 5.15 ± 0.52b | 247.5 ± 2.97c | 411.3 ± 10.3b | 21.3 ± 1.23a |
| S3            | 7.71 ± 0.04b | 2.88 ± 0.28c | 0.10 ± 0.02b | 4.23 ± 0.55a | 3.40 ± 0.35a | 7.99 ± 0.08a | 790.4 ± 7.22a | 882.5 ± 78.5a | 24.5 ± 1.13a |
| S4            | 7.63 ± 0.03ab| 4.73 ± 0.47b | 0.11 ± 0.01b | 4.10 ± 0.51a | 0.53 ± 0.15b | 1.12 ± 0.57d | 367.9 ± 6.52b | 258.2 ± 33.0c | 10.8 ± 2.01b |
| S5            | 7.78 ± 0.02a | 4.15 ± 0.55b | 0.09 ± 0.01b | 3.65 ± 0.66a | 0.72 ± 0.16b | 2.34 ± 0.18c | 333.2 ± 45.3b | 177.6 ± 15.3d | 12.3 ± 0.85b |

Data are mean ± SE (n = 3); different letters in the same column indicate statistically significant differences (SNK test, P < 0.05). WC soil gravimetric water content; TN total nitrogen, SOC soil organic carbon.

Metal concentrations in *P. vittata* roots

Concentrations of Cu, Cd, Zn, Pb, and As in the *P. vittata* roots varied significantly (P < 0.05; Table 2). Concentrations of Cu, Cd, Pb, and As were highest at S2, while the Zn level was highest at S3. Compared with the control site, the Cu, Cd, Zn, Pb, and As concentrations were 2.7 to 6.2 (S2), 2.44 to 6.59 (S3), 0.77 to 6.27 (S4), and 2.04 to 6.34 (S5) times higher, respectively.

Diversity and phylogeny of endophytic bacteria

A total of 116 As-resistant endophytic isolates were obtained. To estimate the diversity, the 16S rRNA genes of the isolates were analyzed by ARDRA, resulting in 16 different ARDRA patterns (Table 3). The 46 representative isolates, selected based on colony morphology and ARDRA, carried 16S rRNA genes 99–100% similar with the reference sequences in GenBank. The representative isolates belonged to five genera in four families: *Alphaproteobacteria* (17 isolates, one genus), *Gammaproteobacteria* (eight isolates, two genera), *Actinobacteria* (three isolates, one genus), and *Firmicutes* (18 isolates, one genus) (Fig. 1). Fifteen of the isolates were obtained from the slag heap site (S5), 12 from the tailing dam site (S5), 12 from the ore charge heap site (S5), and three from the control site (S1) (Fig. 1). Most of the isolates from the slag heap site belonged to the genus *Agrobacterium*. The majority of the isolates from the tailing dam site, the ore charge heap site, and the ore outlet site belonged to the genus *Bacillus*, while the isolates from the control site belonged to the genera *Rhodococcus* and *Agrobacterium*.

Arsenite resistance levels and plant growth-promoting properties of the isolates

In the minimum inhibitory concentration (MIC) test for arsenite, all strains grew in 15 mM, 36 in 36 mM, 19 in 42 mM, and 5 in 48 mM NaAsO₂ (Fig. 1). All 17 *Agrobacterium tumefaciens* isolates showed high MICs (≥ 30 mM), while the *Rhodococcus* sp. isolates showed low MICs (≥ 15 mM). Among the *Pseudomonas* isolates, the isolates containing *arsB* showed higher MICs than those without *arsB*. The average MIC of the 10 *Acr3(2)*-carrying isolates (40.9 ± 4.5 mM) was higher than that of the five *arsB*-carrying isolates (25.8 ± 2.3 mM). Five of the 19 highly arsenite-resistant isolates (MIC > 42 mM) were obtained from *P. vittata* roots with high arsenic levels (Fig. 1, Table 2). Isolates from the control site showed lower average MIC than the other isolates (Fig. 1).

In total, 28 out of the 46 isolates produced both IAA and siderophores (Table 3). The majority of the isolates produced 17.7–103.9 μg ml⁻¹ IAA. Isolates L8 (*Rhizobium* sp.), L11 (*Rhodococcus equi*), L31 (*Agrobacterium tumefaciens*), and X10 (*Bacillus megaterium*) produced over 90 μg ml⁻¹ of IAA. Two-thirds of the sequenced endophytes produced siderophores. Isolates L8 (*Rhizobium* sp.), K5 (*Bacillus subtilis*), and X4 (*Bacillus* sp.) produced more siderophores than the other isolates.

Horizontal transfer of the arsenite transporter genes *arsB* and *Acr3(2)*

A total of 16 arsenite transporter genes, including five *arsB* genes and 11 *ACR3(2)* genes, were successfully amplified (Fig. 2). In the sequence analyses with the BlastX algorithm, the *arsB* and *Acr3(2)* formed separate clusters (Fig. 2). The *arsB* and *Acr3(2)* sequences of the
| Isolate (closest relative sequence) | % identity | ARDRA type | IAA ($\mu$g ml$^{-1}$)$^a$ | Siderophore$^b$ |
|-----------------------------------|------------|------------|-----------------|----------|
| L9 (Agrobacterium tumefaciens)     | 99         | I          | 46.6 ± 4.36     | –        |
| L17 (Agrobacterium tumefaciens)   | 99         | I          | 95.0 ± 4.88     | –        |
| L25 (Agrobacterium tumefaciens)   | 99         | I          | 42.5 ± 9.42     | +        |
| L31 (Agrobacterium tumefaciens)   | 99         | I          | 92.3 ± 8.66     | +        |
| W21 (Agrobacterium tumefaciens)   | 99         | I          | 32.8 ± 2.58     | +        |
| X13 (Agrobacterium rhizogenes)    | 99         | II         | –                | ++       |
| C6 (Agrobacterium rhizogenes)     | 99         | II         | 81.2 ± 4.77     | ++       |
| Y2 (Agrobacterium rhizogenes)     | 99         | II         | 41.4 ± 3.33     | –        |
| K4 (Agrobacterium sp.)            | 99         | III        | 44.4 ± 6.52     | +++      |
| K16 (Agrobacterium sp.)           | 99         | III        | 44.2 ± 5.51     | +        |
| X3 (Agrobacterium sp.)            | 99         | III        | 44.5 ± 7.88     | +        |
| L24 (Agrobacterium sp.)           | 99         | III        | 46.0 ± 2.22     | –        |
| W24 (Agrobacterium sp.)           | 99         | III        | –                | –        |
| L19 (Agrobacterium sp.)           | 99         | III        | 47.6 ± 5.54     | +        |
| L4 (Rhizobium sp.)                | 99         | IV         | 34.8 ± 3.11     | +        |
| L5 (Rhizobium sp.)                | 99         | IV         | 60.9 ± 9.66     | +++      |
| L8 (Rhizobium sp.)                | 99         | IV         | 103.9 ± 11.2    | ++++     |
| W1 (Stenotrophomonas rhizophila)  | 99         | V          | 47.8 ± 4.75     | ++       |
| W22 (Stenotrophomonas sp.)        | 99         | VI         | 204 ± 3.36      | –        |
| L20 (Pseudomonas sp.)             | 100        | VII        | 34.4 ± 2.28     | –        |
| X8 (Pseudomonas sp.)              | 100        | VII        | 120.3 ± 9.61    | +        |
| L6 (Pseudomonas arylphaptanthins) | 99         | VIII       | 45.9 ± 3.25     | +        |
| L1 (Pseudomonas arylphaptanthins) | 99         | VIII       | 32.4 ± 4.44     | ++       |
| W28 (Pseudomonas putida)          | 99         | IX         | 38.0 ± 4.25     | ++       |
| L3 (Pseudomonas putida)           | 99         | IX         | 55.1 ± 4.01     | ++       |
| C1 (Rhodococcus sp.)              | 99         | X          | 29.5 ± 3.15     | ++       |
| C3 (Rhodococcus sp.)              | 99         | X          | 65.6 ± 5.55     | –        |
| L11 (Rhodococcus equi)            | 99         | XI         | 98.8 ± 8.16     | +++      |
| K17 (Bacillus indicus)             | 99         | XII        | –                | +        |
| W12 (Bacillus cereus)             | 99         | XIII       | 49.2 ± 2.17     | +        |
| X2 (Bacillus muralis)             | 99         | XIV        | 22.9 ± 5.43     | –        |
| K5 (Bacillus subtilis)            | 99         | XV         | 31.7 ± 4.22     | ++++     |
| W9 (Bacillus megaterium)          | 99         | XV         | 34.6 ± 1.78     | ++       |
| W23 (Bacillus megaterium)         | 99         | XV         | –                | +        |
| X10 (Bacillus megaterium)         | 99         | XV         | 99.6 ± 12.7     | +++      |
| K2 (Bacillus sp.)                 | 99         | XVI        | 53.1 ± 4.33     | ++       |
| K3 (Bacillus sp.)                 | 99         | XVI        | 43.6 ± 3.16     | –        |
| K12 (Bacillus sp.)                | 100        | XVI        | –                | –        |
| K6 (Bacillus sp.)                 | 100        | XVI        | 81.4 ± 2.44     | ++       |
| W8 (Bacillus sp.)                 | 99         | XVI        | 56.0 ± 2.08     | +++      |
| W10 (Bacillus sp.)                | 99         | XVI        | 54.6 ± 7.03     | +++      |
| W11 (Bacillus sp.)                | 99         | XVI        | –                | –        |
| X4 (Bacillus sp.)                 | 99         | XVI        | 39.0 ± 4.21     | +++      |
| X11 (Bacillus sp.)                | 99         | XVI        | 17.7 ± 3.22     | –        |
Table 3  Plant growth promoting characteristics of endophytic bacterial isolates from *P. vittata* L. (Continued)

| Isolate (closest relative sequence) | % identity | ARDRA type | IAA (μg ml\(^{-1}\))\(^{a}\) | Siderophore\(^{b}\) |
|------------------------------------|------------|------------|----------------|-----------------|
| K9 (Bacillus sp.)                  | 99         | XVI        | 14.9 ± 0.31     | ++              |
| W3 (Bacillus sp.)                  | 99         | XVI        | 15.2 ± 0.90     | +               |

\(^{a}\)IAA production: -= not detectable; \(^{b}\) Siderophore production: +, little; ++, low; +++, moderate; ++++, high

---

![Diagram of bacterial relationships](image)

**Fig. 1** Relationships between the representative isolates and the reference strains based on 16S rRNA gene (~1500 bp) phylogenetic analysis. Minimum inhibitory concentration (MIC) for arsenite and the presence of arsenite-resistance genes *ArsB*/*Acr3(2)* are indicated in squared brackets. Sequences from this study are in bold type. Bootstrap values over 50% are shown on the branching points. The scale bar indicates 2% nucleotide sequence substitution. Isolates with capital L were isolated from the *P. vittata* roots collected from site S5, with X from site S4, with W from site S3, with K from site S2, and with C from site S1.
isolates were similar to those from Gammaproteobacteria and Alphaproteobacteria, respectively. The Acr3(2) sequences were divided into separate subgroups, similar to either Acr3(2) from Agrobacterium sp. or from Ensifer sp. Agrobacterium sp. L5, L8, L9, and L19 carried Acr3(2) similar to those of Ensifer sp. (Fig. 3). Gammaproteobacterial Stenotrophomonas sp. W22 carried Acr3(2) (Fig. 3). The Bacillus sp. K6 carried arsB similar to those of the Gammaproteobacteria clade (Fig. 3). All of these, possibly horizontally transferred ACR3(2) and arsB genes, were obtained from isolates from the highly As-contaminated mining area.

Discussion
Plants that accumulate As can provide a specific environment for bacterial endophytes to adapt to high As concentrations [28]. In this work, we isolated and characterized bacterial endophytes from the roots of P. vitata, an arsenic hyperaccumulator growing in the Pb-Zn mine tailings in Southwest China [18].

In our study, we isolated 116 As-resistant bacterial endophytes. Despite the small number of isolates, the As-resistant bacterial endophyte community was diverse. The representative isolates were affiliated with Pseudomonas, Stenotrophomonas, Rhodococcus, Agrobacterium, and Bacillus. Of these, Agrobacterium, Bacillus, and Pseudomonas species have previously been reported as metal-resistant endophytes in other As-contaminated sites [29–31]. Arsenite-resistant Stenotrophomonas strains have been isolated from As-contaminated soil and water [32], but, to our knowledge, endophytic arsenite-resistant Stenotrophomonas and Rhodococcus have not been isolated earlier. Most of the representative isolates belonged to Agrobacterium (phylum Proteobacteria) and Bacillus (phylum Firmicutes). Similarly, Ni-resistant bacterial endophytes from Alyssum bertolonii belonged mainly to Firmicutes and Actinobacteria [33]. Moreover, 14 endophytes isolated from Sedum alfredii belonged mainly to Proteobacteria (57%) and Firmicutes (43%) [34]. Taken together, these results suggest that the predominant bacterial endophytes in heavy metal-accumulating plants belong to Firmicutes, Proteobacteria, and Actinobacteria [35]. These highly adapted groups may accelerate the degradation of complex soil compounds by producing extracellular enzymes, and thus stimulate the growth of other bacteria in As-contaminated rhizosphere soil [36].
Endophytic bacteria can increase the growth and development of plants growing in contaminated sites and their metal resistance by producing the phytohormone IAA and siderophores [37–39]. Siderophores are organic molecules with a high affinity for Fe(III) ions and form complexes with other metals, thus participating in nutrient mobilization and metal availability to plants [40, 41]. Bacterial siderophore production may be stimulated by heavy metals and can alleviate the toxicity of heavy metals to plants by increasing the supply of iron [41]. Arsenic-resistant strains isolated from *P. vittata* have been reported to produce siderophores [39]. Most of the isolates in our study produced IAA or siderophores, indicating that the endophytes may help their host plant to colonize As-contaminated sites [38].

In our study, *P. vittata* roots from sites S2 and S3 (with high As-concentrations) hosted a higher diversity of arsenite-resistant bacteria. In addition, the resistance levels of the isolates were also higher than those among isolates from sites with intermediate and low As concentrations. The 19 strains with arsenite MICs over 40 mM were all isolated from *P. vittata* growing in the Pb-Zn mining area. Previous studies have proposed that high As-contamination levels are likely to exert a strong selective pressure, thereby decreasing microbial diversity [17, 42]. However, in our study, *P. vittata* was widely distributed in the Pb-Zn mine area [18], which may result in the evolution of more bacterial species that are well adapted to highly arsenic-contaminated *P. vittata*.

Moreover, Huang et al. [43] reported that in a long-term field site (1951-present), arsenite-resistant endophytic microbial communities of *P. vittata* had had sufficient time to adapt to metal and/or metalloid stress. Zhu et al. [35] found that the diversity of As-resistant endophytes in soil with high arsenic levels was higher than that in less contaminated soil. These results suggest that endophytic bacteria have adapted to high arsenic stress levels and maintained their diversity in *P. vittata* after long-term exposure to high As-levels [44].

Exploring the relations among the degree of arsenite resistance, the distribution and diversity of the arsenite transporter gene families of the bacterial endophytes is a key goal of microbial ecology. The *ACR3(2)* genotypes were previously reported to be predominant over *arsB* in bacterial strains isolated from As contaminated soil [17], which was similar to findings in our study. To cope with arsenic toxicity, multiple sets of arsenic resistance genes and operons were developed in the genome of nearly every bacterial species sequenced to date. For example, the full genome sequence of strain *Brevibacterium linens* AE038–8 contained three *ars* operons (*arsC, ACR3* and *arsR*) and two copies of the *arsO* gene [45], *Thiomonas* sp. possessed two operons (*aio* and *ars* system) [46], while *Rhodopseudomonas palustris* CGA009 carried three sets of arsenic resistance determinants (*ars1, ars2, and ars3*) on the chromosome [47]. Previous study has reported that soil bacteria could acquire multiple resistance determinants via chromosomal
duplication or horizontal gene transfer, allowing them to cope with long-term arsenic toxicity [44]. Surprisingly, in our study, none of the isolates carried both types of arsenite transporter genes, even though both these genes are commonly found on the same operon. The ACR3 may have a higher affinity to arsenite and a higher rate of arsenite transport than arsB, which makes it more effective [44]. Accordingly, compared to the arsB gene-containing strains, strains having ACR3(2) mostly showed higher arsenite resistance in our study.

Horizontal gene transfer (HGT) plays an important role in allowing a microbial community to rapidly adapt to a new environmental stress like heavy metal contamination, and thus could play an important role in the adaptation of the endogenous endophytic community [39]. In our present study, ACR3(2) appeared to be more easily transferred than arsB, and the transfer would be possibly stimulated by the exponential growth of environmental pollution as proposed in a previous study [48]. Cai et al. [44] have reported that arsenite transporter genes were transferred between Aeromonas, Stenotrophomonas, and Comamonas in highly As-contaminated soils. Similarly, the phylogenetic discrepancies between 16S rRNA genes and ACR3(2)/arsB indicate that ACR3(2) might have been horizontally transferred, especially in the isolates from P. vittata roots containing high As-levels. The HGT process may have occurred under the high arsenic pressure and resulted in increased functional and species diversity [49, 50], which may have practical applications in equipping the natural endophyte populations capable of resisting As and does not require long-term establishment of the inoculant strain.

Conclusions

We investigated the distribution and diversity of arsenite-resistant endophytic bacteria in P. vittata roots, collected from five soils with different levels of As-contamination, and studied the arsenite resistance and arsenic transport genes of the isolates. Proteobacteria, Actinobacteria, and Firmicutes were the predominant taxa. The distribution and diversity of cultivable endophytes were affected by the arsenic concentration in P. vittata. A high number of the isolates were resistant to high concentrations of As and multiple heavy metals and showed plant growth-promoting characteristics. Horizontal gene transfer of ACR3(2) and arsB was detected in some of the isolates from P. vittata roots with high As concentrations. Overall, this study provides valuable information about endophytic bacterial species in relation with As transport and As-resistance genes. Our results contribute to the knowledge on the diversity and distribution of As-resistant endophytic bacteria that may be applied in the phytoremediation of As-contaminated sites.

Abbreviations

ARDRA: Amplified 16S rDNA restriction fragment analysis; As: Arsenic; CAS: Chrome azurol-S; Cd: Cadmium; CDM: Chemically defined medium; Cu: Copper; DNA: Deoxyribonucleic acid; IAA: Indole-3-acetic acid; LB: Luria–Bertani; MICS: Minimum inhibitory concentration; NCBi: National center for biotechnology information; Pb: Lead; PCR: Polymerase chain reaction; pH: Post hatch; RNA: Ribosomal ribonucleic acid; SLP: Salt low phosphate; SOC: Soil organic carbon; TN: total nitrogen; WC: Water content; Zn: Zinc

Acknowledgements

The authors would like to thank Menglin Chen, Dingjian Pan, Qiaoling Lu, and Jingpeng Luo for the excellent technical support.

Funding

This work was supported by the Natural Science Foundation of China (grant number 41201256). Besides funding the funding bodies played no role in study design, collection of data, or manuscript preparation.

Availability of data and materials

Sequences obtained in this study were deposited in the NCBI GenBank database with accession numbers MF185755–MF185785 for 16S rRNA genes, MF185786–MF185798 for arsB, and MF185799–MF185803 for ACR3(2).

Authors’ contributions

YFG designed the experiments and wrote the manuscript. YHS conducted the experiment. YYW analyzed the basic soil and plant physicochemical data. XMY constructed the phylogenetic tree. KZ and QJX participated in the initial draft and the revision of the manuscript. XPZ coordinated the study. Designed portions of the experiments. QC revised the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 31 October 2017 Accepted: 30 April 2018

Published online: 08 May 2018

References

1. Ahernathy CO, Thomas DJ, Calderon RL. Health effects and risk assessment of arsenic. J Nutr. 2003;133:1536–8.
2. Zhao FJ, McGrath SP, Meharg AA. Arsenic as a food chain contaminant: mechanisms of plant uptake and metabolism and mitigation strategies. Annu Rev Plant Biol. 2010;61:535–9.
3. XiaoXY, CTB, Xie H, Liu YR. Soil as contamination and its risk assessment in areas near the industrial districts of Chenzhou City, southern China. Environ Int. 2005;31:791–8.
4. Gomes MP, Carvalho M, Carvalho GS, Marques TC, Garcia QS, Guerlheimer LRQ, Soares AM. Phosphorus improves arsenic phytoaccumulation by Anadenanthera peregrina by alleviating induced oxidative stress. Int J Phytorem. 2013;15:633–6.
5. Shin MN, Shim J, You Y, Myung H, Bang KS, Cho M, Kamala-Kannan S, Oh BT. Characterization of lead resistant endophytic bacillus sp. MN-4 and its potential for promoting lead accumulation in metal hyperaccumulator Alnus firma. J Hazard Mater. 2012;199:314–20.
6. Słonecka T, Haack B, Price S. Spectroscopic analysis of arsenic uptake in Pteris F. Rem S. 2009;164:4–5.
7. Ma LQ, Konar KM, Tu C, Zhang WH, Cai Y, Kesselley ED. A fern that hyperaccumulates arsenic. Nature. 2001;409:579.
8. Weyens N, van der Lelie D, Taghavi B, Vangronsveld. J. Phytoremediation: plant–endophyte partnerships take the challenge. Curr Opin Chem Biol. 2009;20:248–4.
9. Oremland RS, Stolz JF. Arsenic, microbes and contaminated aquifers. Trends Microbiol. 2005;13:45–9.
Gu et al. BMC Microbiology (2018) 18:42

10. Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN. Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett. 2008;278:1–9.
11. Khan Z, Doty SL. Characterization of bacterial endophytes of sweet potato plants. Plant Soil. 2009;322:197–7.
12. Ulrich K, Ulrich A, Ewald D. Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiol Ecol. 2008;63:169.
13. Berg G, Köberl M, Rybakova D, Müller H, Gerosch R, Smalla K. Plant microbial diversity is suggested as the key to future biocatalyst and health trends. FEMS Microbiol Ecol. 2017; https://doi.org/10.1093/femsec/fix050.
14. Luo S, Wan Y, Xiao X, Guo HJ, Chen L, Xi Q, Zeng GM, Liu CB, Chen JL. Isolation and characterization of endophytic bacterium LB07 from cadmium hyperaccumulator Solanum nigrum L. and its potential for remediation. Appl Microbiol Biotechnol. 2011;88:1637–7.
15. Silver S, Phung LT. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl Environ Microbiol. 2005;71:599–608.
16. Bhattacharjee H, Rosen BP. Arsenic metabolism in prokaryotic and eukaryotic microbes. Mol Microbiol. Heavy Metals. 2007;6:371–6.
17. Achor AR, Bauda P, Billard P. Diversity of arsenite transporter genes from ars-resistant soil bacteria. Res Microbiol. 2007;158:128–7.
18. Chen HL, Zhang SR, Li T, Dai Y, Mao Z, Heavy- metal accumulation and tolerance of plants at zinc- lead mine tailings in Hanyuan. J Agro-Environ Sci. 2007;26:505–9. (In Chinese)
19. Gallego JR, Esquinas N, Rodríguez-Valdés E, Menéndez-Aguado JM, Sierra C. Characterization of bacterial endophytes of sweet potato plants. Plant Soil. 2009;322:197–7.
20. Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of physiological, taxonomic, and molecular characteristics of a cultivable arsenic-resistant bacterial community. Environ Sci Pollut Res Int. 2015;22:13753–3.
21. Chang JS, Yoon IH, Kim KW. Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant Alyssum bertolonii. Micro Ecol. 2007;53:305–6.
22. Barzanti R, Ozio F, Bazzicalupi M, Gabbrielli R, Galardi F, Bonelli C, Mengoni A. Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant Alyssum bertolonii. Micro Ecol. 2007;53:305–6.
23. Long XX, Chen YM, Chen YG, Woon-Chung WJ, Wei ZB, Wu QT. Isolation and characterization endophytic bacteria from hyperaccumulator Sedum alfredi Hance and their potential to promote phytoextraction of zinc polluted soil. World J Microbiol Biotechnol. 2011;27:1197–7.
24. Zhu Li, Guan DX, Luo J, Rathinasabapathi B, Ma LQ. Characterization of arsenic-resistant endophytic bacteria from hyperaccumulators Pteris vittata and Pteris multifida. Chemosphere. 2011;4:1139–6.
25. Fiener N, Lauber CL, Ramírez KS, Zaneveld J, Bradford MA, Knight R. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J. 2012;6:1007–7.
26. Ali S, Duan J, Charles TC, Blick BR. A bioinformatics approach to the determination of genes involved in endophytic behavior in Burkholderia spp. J Theor Biol. 2014;343:193–8.
27. Tiwari S, Sanagi BK, Thul ST. Identification of arsenic resistant endophytic bacteria from Pteris vittata root and characterization for arsenic remediation application. J Environ Manag. 2016;180:359–56.
28. Taghavi S, Barac T, Greenberg B, Borremans B, Vangronsveld J, van der Lelie D. Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phyto remediation of toluene. Appl Environ Microbiol. 2005;71: 8500–5.
29. Rajkumar M, Ne P, Prasad MNV, Freitas H. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol. 2010;28:142–9.
30. Schalk L, Kanniev M, Breda A. New roles for bacterial siderophores in metal transport and tolerance. Environ Microbiol. 2011;13:2844–4.
31. Jackson CR, Harrison KG, Dugas SL. Enumeration and characterization of cultivable arsenate resistant bacteria in a large estuary. Syst Appl Microbiol. 2005;28:727–4.
32. Huang A, Teplitski M, Rathinasabapathi B, Ma L. Characterization of arsenic-resistant bacteria from the rhizosphere of arsenic hyperaccumulator P. vittata. Can J Microbiol. 2010;56:236–46.
33. Cai L, Liu GH, Rensong C, Wang GJ. Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. Microbiol. 2009;94.
34. Maizel D, Blum JS, Ferrero MA, Uetturkar SM, Brown SD, Rosen BP, Oremland RS. Characterization of the extremely arsenic-resistant Brevibacterium linens strain AE038-8 isolated from contaminated groundwater in Tucumán, Argentina. Int Biodeterior Biodegrad. 2016;107:46–7.
35. Arslêne-Ploetzke F, Koechler S, Marchal M, Coppée JY, Chandler M, Bonnefoy V, Brochier-Armanet C, Barakat M, Barbe V, Battaglia-Brunet F, Brunée O, Bryan CG, Cleis-Arnold J, Cruveiller S, Erhardt M, Heinrich-Salmeron A, Hommais F, Joulian C, Kim E, Lieutaud A, LiÉVemont D, Michel C, Muller D, Otert P, Proux C, Siguer F, Roche D, Rouy Z, Salvigdor G, Slemetry D, Talla E, Weiss S, Weissenbach J, ÊDigue C, Bertin PN. Structure, function, and evolution of the Thermos spp. Genome PLoS Genet. 2006;6:1000859.
36. Zhao CG, Zhang Y, Chen ZH, Chen SC, Yang SP. Insights into arsenic multiple-operators expression and resistance mechanisms in Rhodopseudomonas palustris CAS-009. Front Microbiol. 2015;6:986.
37. Cavalcá L, Zanchi R, Corsini A, Colombo M, Romagnoli C, Carosi E, Andreoni V. Arsenic-resistance bacteria associated with roots of the wild Crinum enervum (L.) plant from an arsenic polluted soil, and screening of potential plant growth-promoting characteristics. Syst Appl Microbiol. 2010;33:154–6.
38. Jackson CR, Dugas SL. Phylogenetic analysis of bacterial and archaeal arsenC gene sequences suggests an ancient, common origin for arsenate transport and tolerance. Environ Microbiol. 2010;12:158.
39. Cavalcá L, Zanchi R, Corsini A, Colombo M, Romagnoli C, Carosi E, Andreoni V. Arsenic-resistance bacteria associated with roots of the wild Crinum enervum (L.) plant from an arsenic polluted soil, and screening of potential plant growth-promoting characteristics. Syst Appl Microbiol. 2010;33:154–6.
40. Jackson CR, Dugas SL. Phylogenetic analysis of bacterial and archaeal arsC gene sequences suggests an ancient, common origin for arsenate resistance. BMC Evol Biol. 2003;3:18.
41. Richard D, Ravigné V, Rieux A, Facon B, Boyer C, Boyer K, Gaygèl P, Javigny S, Teyville M, Canteros BI, Robeléen VG, Chabriand A, Pruvoit O, Lefèvre P. Adaptation of genetically monomorphic bacteria: evolution of copper resistance through multiple horizontal gene transfers of complex and versatile mobile genetic elements. Mol Ecol. 2017;26:2131–9.