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Genome Analysis Revealing the Potential Mechanisms for the Heavy Metal Resistance of *Pseudomonas* sp. P11, Isolated from Industrial Wastewater Sediment

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Abstract
Pseudomonas sp. P11 was isolated from the industrial wastewater sediment nearby the Daye Non-ferrous Metals Company, China. This strain possesses the ability to resist various heavy metals and efficiently precipitate arsenic. We here present a summary classification and a set of features of *Pseudomonas* sp. P11, together with the description of the genomic sequencing and annotation. The genomic sequence is 6,644,817 bp with a G+C content of 62.20% and contains 6143 protein-coding genes, 250 pseudo genes, and 76 tRNAs/rRNAs genes. Operons and gene clusters responsible for multiple heavy metal tolerance or detoxification were identified and accounted for the observed resistance phenotypes. Phylogenetic analysis revealed that the paralogous arsenic resistant genes possess different evolutionary paths. This study provides important insights to illuminate the versatility and adaptation of this strain to the heavy metal-contaminated environment.

Introduction

The genus *Pseudomonas* belongs to the family *Pseudomonadaceae* was originally described by Migula in 1894 [18, 26]. Members of this genus were isolated from diverse environments including soil, sediment, water, air, plants, and clinical specimens. Some of *Pseudomonas* species were also isolated from contaminated niches and possess chromosomal, transposon, and plasmid-mediated resistance systems to adapt the long-term exposure to toxic xenobiotics [20]. For example, *Pseudomonas aeruginosa* isolates carry a high frequency of heavy metal resistance genes, such as *copA* and *copB* genes which are associated with resistance to copper, *czcA* gene which is related to resistance to the metals cadmium, zinc, and cobalt, and *arsC* gene encoding the arsenate reductase [9, 21]. *P. putida* isolates possess a large variety of determinants involved in metal(loid) homeostasis, tolerance and resistance in the genome, including the *nikRABCDE* putative operon that mediates uptake of nickel ions, the *cusCFBA* operon that determines an efflux pump for copper and silver ions, the arsenate resistance operon (*ars*), and the *czc* determinant for cobalt–zinc–cadmium resistance [1, 3]. Based on the special metabolic capacity, *Pseudomonas* species have been explored as candidates for the biological remediation of heavy metal-contaminated soil and water [16, 17, 22].

Pseudomonas sp. P11 was isolated from wastewater sediment sample collected from Daye Non-ferrous Metals Company, China. This strain displayed high tolerance to various metal cations including arsenic, cadmium, mercury, copper, and lead, and it is also able to promote arsenic precipitation in the process of growth. In this report, we presented the genome of stain P11 with special emphasis on the genes involving in metal resistance and detoxification. These data will improve our understanding on the resistance mechanism of heavy metal in strain P11 and thus help make it a better use in the bioremediation field.

Materials and Methods

Sample Collection and Bacteria Isolation

A sediment sample was collected from a wastewater reservoir in Daye Non-ferrous Metals Company, China. 1.0 g of

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sediments were serially diluted and plated onto 0.1 × tryptic soy broth (TSB; Difco) agar plate containing 5 mM arsenate. The plate was incubated at 30 °C for 2 days. Bacterial strains with different morphological characteristics were isolated and purified. The purified strains were stored in glycerol stocks at − 80 °C until further use.

Phenotypic Analysis

Bacterial morphology and motility were observed under a phase contrast microscope. Gram-staining was performed as described previously [23]. Bacterial growth at different temperatures (4, 10, 20, 28, 30, 35, 37, 42 °C) and different pH values (5.0–11.0) were examined. Salt tolerance was also determined by growing the bacteria in 0.1 × TSB medium containing different concentrations of NaCl (0–10%, w/v), respectively. Oxidase activity, catalase activity, and capability to hydrolyze starch, cellulose, chitin, casein, and tyrosine were also tested as described previously [23]. Biochemical features were determined using the API kits (API 50CH and API 20E) according to the manufacturer's instruction (BioMerieux, France).

16S rRNA Gene Amplification and Phylogenic Analysis

Genomic DNAs of bacteria were isolated using MiniBEST Bacterial Genomic DNA Extraction Kit Version 2.0 (TaKaRa Biotechnology Co., Tokyo, Japan). 16S rRNA gene was amplified by PCR using the primers 27F and 1492R as described previously [12]. PCR products were gel purified and sequenced by Genscript (Nanjing, China). Pairwise sequence identities of 16S rRNA genes were calculated using the EzBioCloud server (https://www.ezbiocloud.net/) [10]. Sequences of 16S rRNA, *gyrB*, *arsC,* and *aioA* genes were obtained from the GenBank database. Multiple sequence alignment was performed using ClustalW [13]. Phylogenetic trees were constructed using maximum-likelihood method implemented in MEGA7.0 program [11]. The topology of the tree was evaluated using the bootstrap resampling method with 1000 replicates.

Heavy Metal Resistance

The bacterial cells of strain P11 were grown on 0.1 × TSB agar plates amended with different concentrations of heavy metals to determine the minimal inhibitory concentration (MIC). Heavy metals used in different concentrations included Pb (0–5 mM), Cu (0–5 mM), Hg (0–1 mM), As(III) (0–100 mM), As(V) (0–100 mM), Cd (0–5 mM), and Cr(VI) (0–10 mM). The bacterial cells were plated on each metal concentration in duplicate. Positive controls were set by growing the isolate in the absence of heavy metals under the same conditions. The plates were incubated at 30 °C for 7 days and growth was confirmed by the presence of visible colonies.

DNA Extraction and Whole Genome Sequencing

Cells of strain P11 was cultivated at 30 °C in 0.1 × TSB liquid medium to mid-exponential phase. Genomic DNA was extracted from 0.5 to 1.0 g of cells using the modified method of Marmur [15]. The integrity and quality of the DNA was verified using agarose gels and the NanoDrop™ ND-1000 Spectrophotometer (Biolab). The draft genome of *Pseudomonas* sp. P11 was sequenced at the Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) using the Illumina/Solexa HiSeq 2000 technology.

Genome Assembly and Annotation

The de novo genome of strain P11 was assembled using the Short Oligonucleotide Analysis Package (Velvet v1.2.10) with a k-mer of 55 for contig generation and scaffolding [27]. Genes in the whole genome were identified using Glimmer v3.02 [4]. The predicted CDSs were translated into amino acid sequences that were used as queries to BLAST the GenBank, Swissprot, TIGRFam, Pfam, KEGG, COG, and GO databases, respectively. These data were combined to assert a product description for each predicted protein. Additional gene prediction analysis and functional annotation were performed using the National Center for Biotechnology Information Prokaryotic Genomes Annotation Pipeline (PGAP) and the Integrated Microbial Genomes-Expert Review (IMG-ER) platform [2, 24].

Comparative Genome Analysis

Genomic data of six most closely related *Pseudomonas* species, including *P. entomophila* L48^T, *P. monteilii* SB3078^T, *P. mosselii* BS011^T, *P. plecoglossicida* NyZ12^T, *P. soli* LMG 27941^T, and *P. taiwanensis* DSM 21245^T, were obtained from the public database NCBI (https://www.ncbi.nlm.nih.gov/genome/). Together with strain P11, seven extracted protein sequences from genomes were adjusted to a prescribed format and were grouped into homologous clusters using OrthoMCL based on sequence similarity [6]. The BLAST was applied with the criterion of e-value < $1e^{-5}$, identity > 30%, and length coverage of a gene > 50%, and Markov Cluster Algorithms were employed with an inflation index of 1.2 to complete cluster analysis [5, 19]. Enriched KEGG pathway analyses of specific genes in strain P11 were performed by R packages Cluster Profiler (http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html).

Results and Discussion

Phenotypic Features

Phenotypic characterization showed that *Pseudomonas* sp. P11 was a Gram-staining-negative, motile, and non-sporulating bacterium. Cells were rod shaped with rounded ends and formed opalescent, opaque, moist, circular, and entire margin colonies on 0.1 × Trypticase Soy Agar plate. Strain P11 can grow at a wide range of temperatures from 4 °C to 37 °C, with the optimum of 30 °C. It can proliferate in a pH range of 6.0–8.5, and the optimum growth pH is 7.0. The strain is citrate utilization positive and negative for H_2S and indole production. It produces arginine dihydrolase, tryptophane deaminase, ornithine decarboxylase, and ornithase decarboxylase, but not lysine decarboxylase, gelatinase, beta-galactosidase, and urease.

Strain P11 can produce acid from L-arabinose, D-xylose, ribose, galactose, D-fucose, gluconate, and potassium 2-ketogluconate, but not glycerin, D-arabinose, L-xylose, adonitol, glucose, fructose, sorbose, rhamnose, dulcitol, inositol, sorbital, mannital, α-methy-D-lglycoside, α-methy-D-glucoside, *N*-Acetylglucosamine, amygdalin, arbutin, esculin, salicine, cellobiose, maltose, lactose, saccharose, trehalose, synanthrin, loose three sugar, red versicolor alcohol, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, and potassium 5-ketogluconate.

Phylogeny of 16S rRNA and gyrB Gene Sequences

Strain P11 shared 99.79% 16S rRNA gene sequence similarity with *Pseudomonas hunanensis* LV (GenBank accession no. JX545210) and 97.28–99.09% sequence similarity with the type strains of other recognized species of the genus *Pseudomonas*. The phylogenetic relationships based on 16S rRNA sequences also illustrated that strain P11 is a member of *Pseudomonas* genus and most closely related to *Pseudomonas hunanensis* LV, but phylogenetic analysis using *gyrB* gene sequences indicated that strain P11 was closely related to *P. putida* and showed 90% of the similarity to that of the type strain in the genus *Pseudomonas* (Fig. 1). Therefore, we could not make a conclusion about the taxonomy of strain P11. It was referred to as *Pseudomonas* sp. P11 at present. This stain was stored at China Center for Type Culture Collection (collection no. CCTCC M2016700).

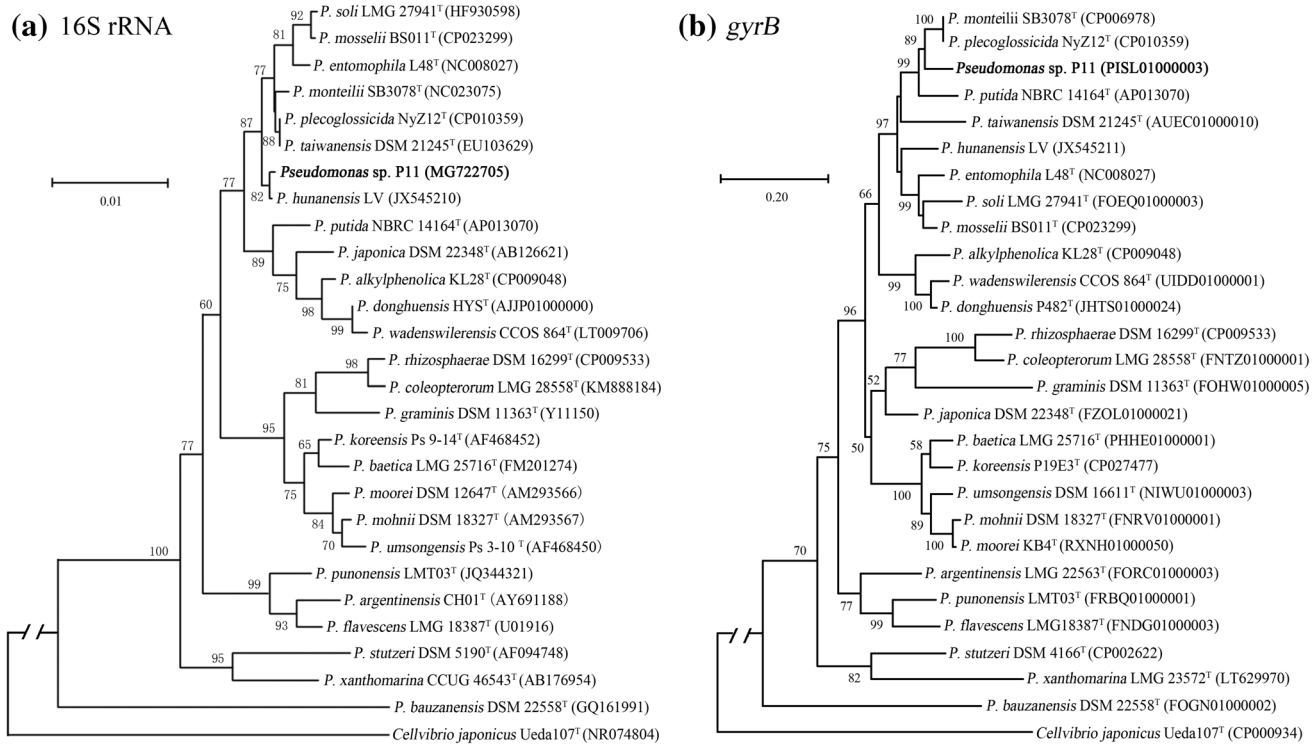

Fig. 1 Phylogenetic tree based on 16S rRNA (**a**) and *gyrB* (**b**) gene sequences showing the phylogenetic position of *Pseudomonas* sp. P11. Sequences were aligned with the Cluster W program and were constructed using maximum-likelihood method implemented in MEGA 7.0 program [11, 13]. GenBank accession numbers are listed in parentheses. Type strains are indicated with a superscript T. Bootstrap support values for 1000 replications above 50% are shown near nodes. The scale bar indicates 0.01 (**a**) and 0.20 (**b**) nucleotide substitution per nucleotide position

Heavy Metal Resistance

Strain P11 displayed resistance to all heavy metal tested during the growth. The MICs reached up to 1.5 mM Pb(II), 1 mM Cu(II), 5.5 mM Hg(II), 40 mM As(III), 70 mM As(V), 3 mM (II), and 2 mM Cr(VI). The relative strong heavy metal resistance of this bacterium suggested that it evolved unique strategies to adapt to the heavy metal stresses.

Table 1 Genomic features of *Pseudomonas* sp. P11

Attribute	Value	% of total
Genome size (bp)	6,644,817	100.00
DNA coding (bp)	5,559,831	83.67
DNA G+C (bp)	4,133,076	62.20
DNA scaffolds	172	100.00
Total genes	6469	100.00
Protein-coding genes	6143	94.96
RNA genes	76	1.17
Pseudo genes	250	3.86
Genes in internal clusters	3463	53.53
Genes with function prediction	4960	76.67
Genes assigned to COGs	4341	67.10
Genes with Pfam domains	4351	67.26
Genes with signal peptides	637	9.85
Genes with transmembrane helices	1378	21.30
CRISPR repeats	5	

Genome Properties and Comparative Genome Analysis

Based on the high tolerance to heavy metals, strain P11 was selected for DNA sequencing. The properties and statistics of the genome are summarized in Table 1. The assembly of the draft genome sequence consists of 172 scaffolds and 6,644,817 bp with 62.20% G+C content. A total of 6469 genes were predicted and of those 6143 were protein-coding genes, 250 were pseudo genes and 76 were tRNAs/rRNAs genes. A total of 4341 (67.1%) predicted proteins were functionally categorized, which allowed for the calculation of the proportions in each COG category (Table 2; Fig. 1) [7]. This Whole Genome Shotgun project has been deposited at GenBank under accession number PISL01000000.

Genome comparison among stain P11 and other 6 *Pseudomonas* strains revealed that these strains shared 3460 gene families (orthologous clusters), and there were 91 unique genes in strain P11 which were assigned to 42 gene families (Fig. 3). Enriched KEGG pathway analysis of these 91 genes showed that the majority of specific genes

Table 2 Number of genes associated with general COG functional categories

Code	Value	%Age	Description
J	168	2.60	Translation, ribosomal structure and biogenesis
A	2	0.03	RNA processing and modification
K	383	5.92	Transcription
L	208	3.22	Replication, recombination and repair
B	2	0.03	Chromatin structure and dynamics
D	37	0.57	Cell cycle control, cell division, chromosome partitioning
V	43	0.66	Defense mechanisms
T	309	4.78	Signal transduction mechanisms
M	246	3.80	Cell wall/membrane biogenesis
N	88	1.36	Cell motility
U	89	1.38	Intracellular trafficking and secretion
O	166	2.57	Posttranslational modification, protein turnover, chaperones
C	290	4.48	Energy production and conversion
G	234	3.62	Carbohydrate transport and metabolism
E	474	7.33	Amino acid transport and metabolism
F	87	1.34	Nucleotide transport and metabolism
H	131	2.03	Coenzyme transport and metabolism
I	171	2.64	Lipid transport and metabolism
P	316	4.88	Inorganic ion transport and metabolism
Q	119	1.84	Secondary metabolites biosynthesis, transport and catabolism
R	331	5.12	General function prediction only
S	447	6.91	Function unknown
–	2128	32.90	Not in COGs

The total is based on the total number of protein-coding genes in the genome

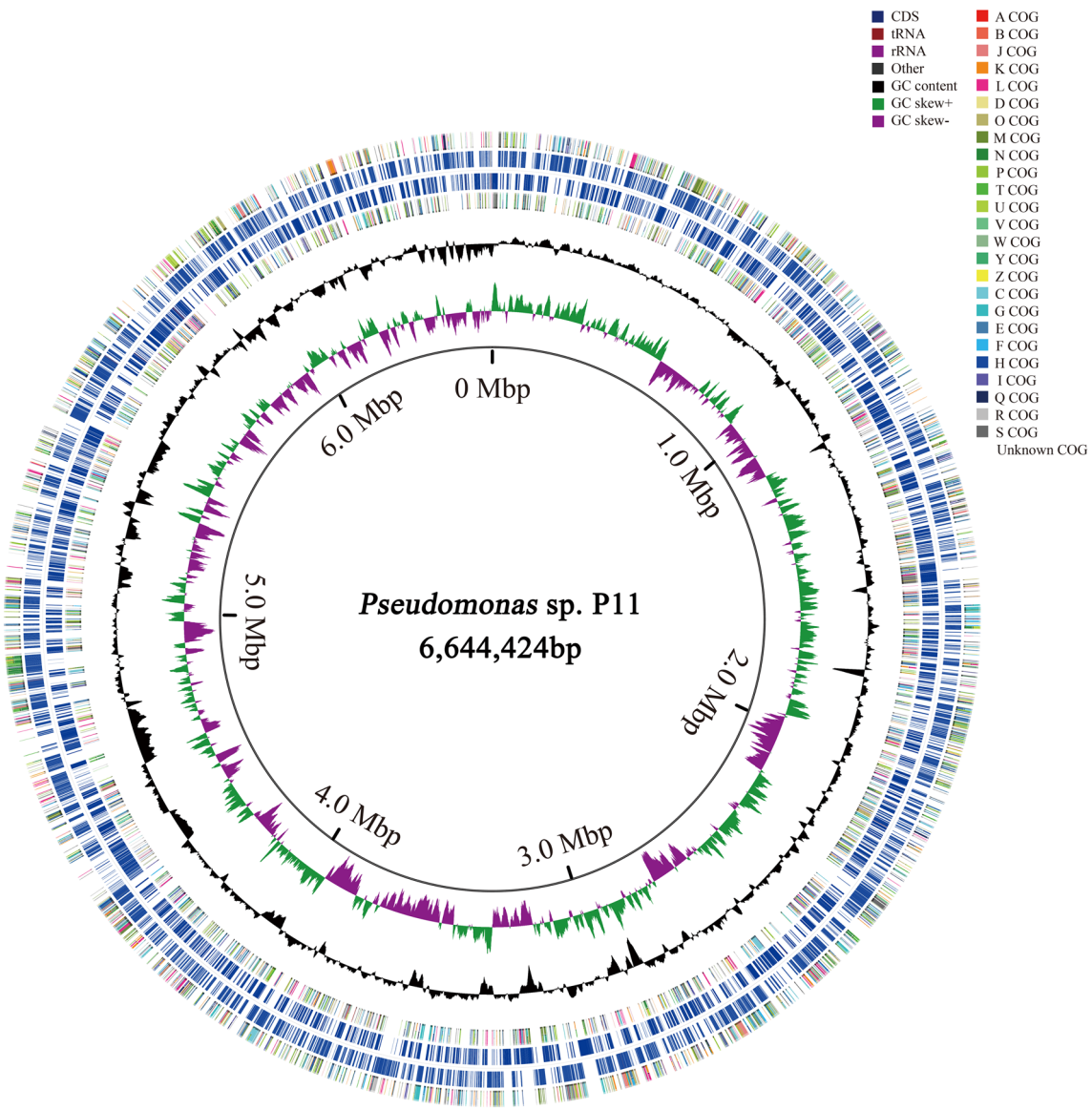

Fig. 2 Circular map of the high quality draft genome of *Pseudomonas* sp. P11 displaying relevant genome features. From outside to center, ring 1 and 4 show protein-coding genes oriented in the forward (colored by COG categories) and reverse (colored by COG categories) directions, respectively. Ring 2 and 3 denote genes on forward/reverse strand; ring 5 shows G+C% content plot, and the innermost ring shows GC skew, purple indicating negative values and olive, positive values. Map was generated using the CGview comparison tool [8] (Color figure online)

in P11 were ABC (ATP-binding cassette) transporters. In bacteria, ABC transporters are of critical importance because they play roles in nutrient uptake and in secretion of toxins and antimicrobial agents. The gene annotation results showed that the genome of *Pseudomonas* sp. P11 possess numerous ABC transporters that mediated the uptake and secretion of nutrients, such as sugar, amino acid, peptide, metal, iron-siderophores, taurine, urea, polyamine, and vitamin ABC transporter.

Genomic Insights of Heavy Metal Resistance

The annotated genomes of *Pseudomonas* sp. P11 revealed the presence of various putative proteins related to multiple heavy metals resistance, including resistance proteins, transporters, and metal reductases (Fig. 4). A total of eight putative proteins conferring copper resistance, homeostasis, or transport were identified in the genome, including copper resistance protein CopB, CopC, and CopD, copper-translocating P-type ATPase CopA, Cu(I)-responsive

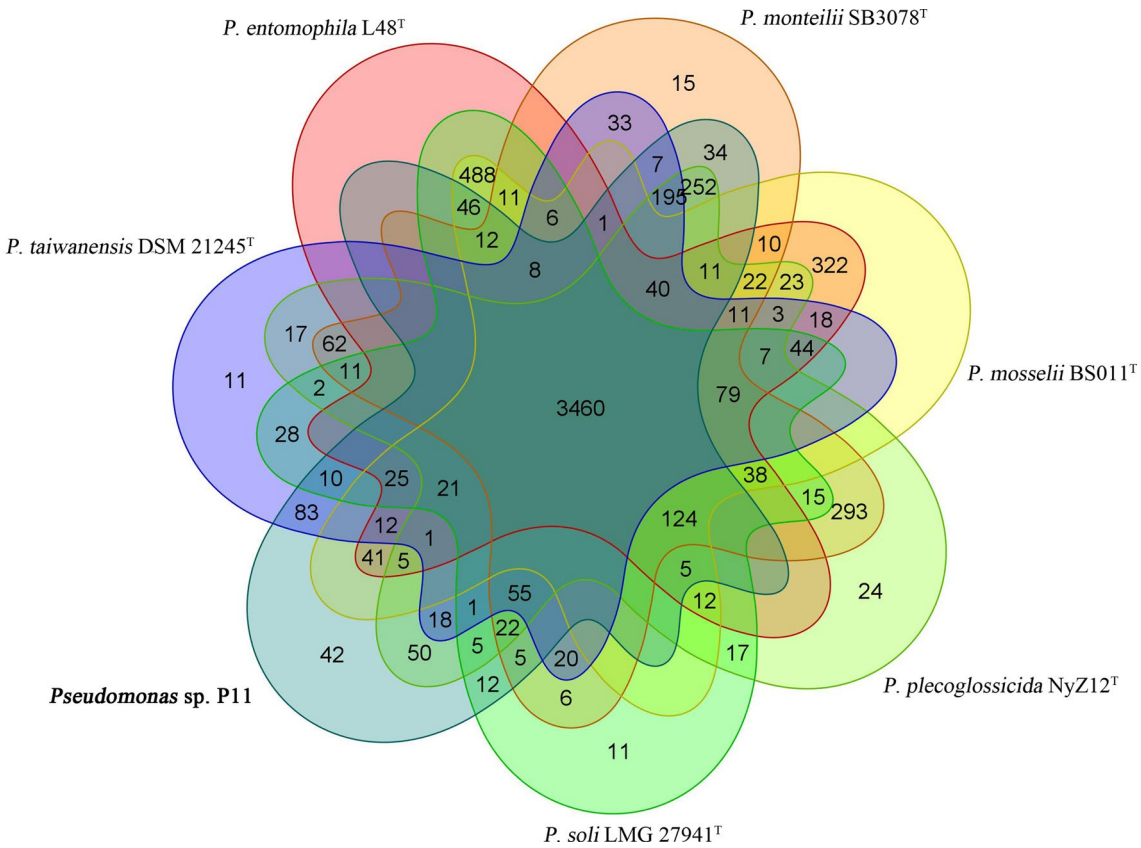

Fig. 3 Venn diagram showing the distribution of shared gene families (orthologous clusters) among *Pseudomonas* sp. P11, *P. entomophila* L48^T, *P. monteilii* SB3078^T, *P. mosselii* BS011^T, *P. plecoglossicida* NyZ12^T, *P. soli* LMG 27941^T and *P. taiwanensis* DSM 21245^T. The cluster number in each component is listed

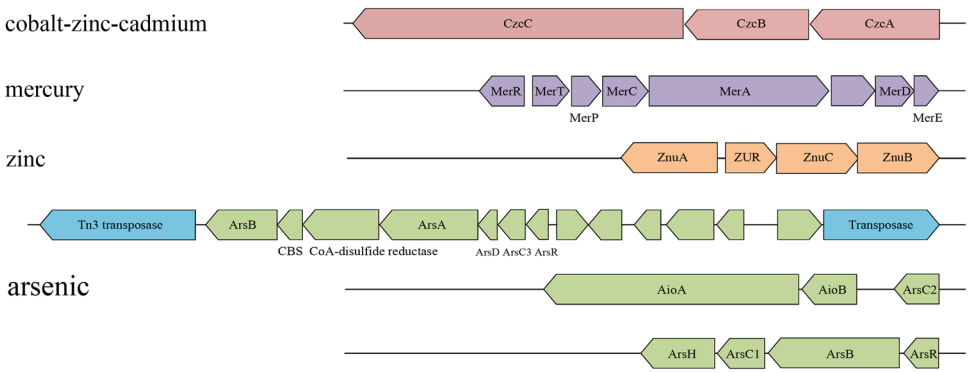

Fig. 4 Product of putative gene clusters and operons involved in metal cation resistance determinants annotated using PGAP. Annotated genes attributed to cobalt–zinc–cadmium, mercury, zinc and arsenic are displayed in purple, orange and olive, respectively (Color figure online)

transcriptional regulator CueR, multicopper oxidase CueO, copper chaperone CupA, and copper transporter CusS. Meanwhile, A *CzcCBA* operon encoding cobalt–zinc–cadmium resistance (czc) determinants, a *ZnuABC* transporter involved in zinc (Zn^{2+}) homeostasis and biotolerance, and a mercury resistance operon consists of seven detoxifying enzyme genes (*MerR*, *MerT*, *MerP*, *MerC*, *MerA*, *MerD,* and *MerE*) were also found in the genome [14, 25].

Strain P11 carries three operons responsible for arsenic resistance and metabolism in the genome. The first operon contains four genes, including *arsR*, encoding the transcriptional repressor; *arsB*, encoding the arsenic efflux pump protein; *arsC*1, encoding the arsenate reductase; and *arsH*, encoding the organoarsenical oxidase. The second operon consists of genes encoding arsenite oxidase large unit AioA, arsenite oxidase small unit AioB and arsenate reductase

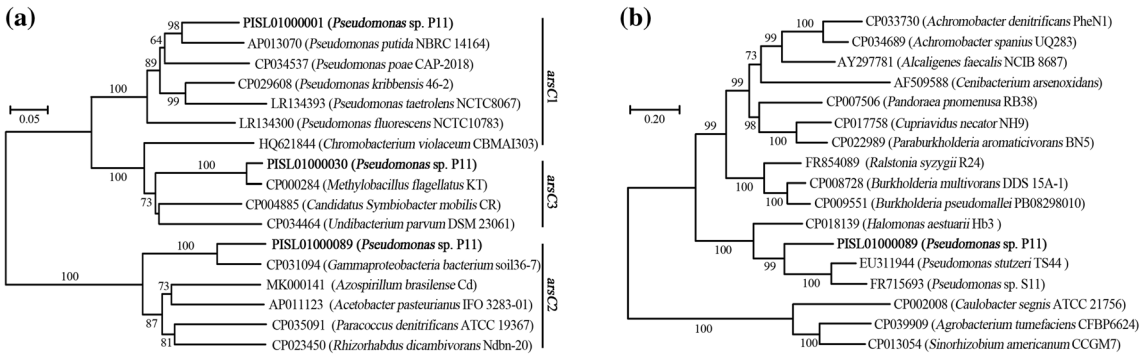

Fig. 5 Maximum-likelihood trees based on *arsC* (**a**) and *aioA* (**b**) gene sequences. Numbers represent percentages of 1000 bootstraps and are only shown for bootstrap values > 60%. The scale bar indicates 0.05 (**a**) and 0.20 (**b**) nucleotide substitution per nucleotide position

ArsC2. The third operon contains five genes, including *arsB*, *arsA*, *arsD* (encoding the arsenic metallochaperone ArsD), *arsC*3, and *arsR*, but in which the *arsB* and *arsA* genes are separated by two genes encoding predicted CBS domain-containing protein and CoA-disulfide reductase. It's worth noting that *arsBADCR* genes were encompassed by two transposase genes in the third operon, suggesting that this arsenic operon in this strain may be acquired by horizontal gene transfer. Previous research has shown that the *ars* operons are quite diverse in *Pseudomonads* such as *P. aeruginosa* and *P. putida* [1, 3, 9, 21]. Sequence analysis indicated that three *ars* operons in *Pseudomonas* sp. P11 were differentiated from previously described *ars* operons in other *Pseudomonas species*. This may imply a new strategy for arsenic metabolism in this strain.

It was widely accepted that arsenic oxidase and resistant reductase genes were horizontally transfered between bacteria species. The phylogenetic trees based on *arsC* and *aioA* gene sequences were constructed to find the potential evolution path of these genes in strain P11 (Fig. 5). The *arsC*1 groups with the thioredoxin-requiring arsenate reductases from *Pseudomonas* species. The *arsC*2 is more closely related to glutaredoxin-requiring arsenate reductases from some species of α-*Proteobacteria*, such as *Azospirillum brasilense*, *Acetobacter pasteurianus*, *Rhizorhabdus dicambivorans*, and *Paracoccus denitrificans*. The *arsC*3 was clustered together with the *arsC*s from the members of *β-Proteobacteria*, including *Chromobacterium violaceum*, *Methylobacillus flagellates*, *Candidatus Symbiobacter mobilis*, and *Undibacterium parvum*. The *aioA* shows high sequence similarity to *aioA* sequences found in *Pseudomonas* species. These results indicated that the *arsC* genes likely have different origins, the *arsC*2 and *arsC*3 could be transferred from *α-Proteobacteria* and *β-Proteobacteria* with the help of gene transfer events, respectively.

In addition, the genome sequence analyses revealed the presence of tellurium resistance protein TerB and TerC, chromate transporter ChrA, chromate reductase, magnesium and cobalt efflux protein CorC, Mg/Co/Ni transporter MgtE, molybdenum cofactor guanylyltransferase MobA, molybdenum ABC transporter ATP-binding protein, selenate ABC transporter, Cd(II)/Pb(II)-responsive transcriptional regulator CadR, cadmium-translocating P-type ATPase CadA, Cobalt ABC transporter CbtK and CbtL, and three heavy metal translocating P-type ATPases.

The presence of these genes in the genome suggest that *Pseudomonas* sp. P11 is adapted to thrive in environments with metal(loid) contamination. Meanwhile, the unique mechanism of heavy metal resistance in this bacterium suggested its great bioremediation potentials in heavy metal-contaminated environment.

Conclusions

Pseudomonas sp. P11 was isolated from industrial wastewater sediment. Our interest in studying the genome of this strain started when we found that this bacterium can tolerate several heavy metals and precipitate arsenic. In this study, we characterized the whole genome of strain P11 and revealed that numerous genes in the genome involved in the tolerance or detoxification of metal cations, including arsenic (As), mercury (Hg), zinc (Zn), cobalt (Co), cadmium (Cd), tellurium (Te), chromate (Cr), and molybdenum (Mo). We highlighted that the paralogous arsenic resistant genes probably possessed different evolutionary paths. The genome sequence provides useful information for discovering the tolerance mechanisms of microorganism in heavy metal-contaminated environment and will drive the use of *Pseudomonas* sp. P11 as a microbial candidate for bioremediation, such as arsenite and chromate.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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