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Isolation and characterization of the heavy metal resistant bacteria CCNWRS33-2 isolated from root nodule of *Lespedeza cuneata* in gold mine tailings in China

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ABSTRACT

A total of 108 strains of bacteria were isolated from root nodules of wild legumes growing in gold mine tailings in northwest of China and were tested for heavy metal resistance. The results showed that the bacterial strain CCNWRS33-2 isolated from *Lespedeza cuneata* was highly resistant to copper, cadmium, lead and zinc. The strain had a relatively high mean specific growth rate under each heavy metal stress test and exhibited a high degree of bioaccumulation ability. The partial sequence of the copper resistance gene *cop*A was amplified from the strain and a sequence comparison with our Cu-resistant PCR fragment showed a high homology with Cu-resistant genes from other bacteria. Phylogenetic analysis based on the 16S rRNA gene sequence showed that CCNWRS33-2 belongs to the *Rhizobium–Agrobacterium* branch and it had 98.9% similarity to *Agrobactrium tumefaciens* LMG196.

1. Introduction

Heavy metal contamination of soil is widespread [1]. Many agricultural and industrial practices have led to environmental pollution by heavy metal ions. Heavy metal contamination in soil comes from anthropogenic sources, such as smelters, mining, power stations and the application of pesticides containing metal, fertilizer and sewage sludge [2–4]. Heavy metal contamination can have significant effects on indigenous microbial populations. For example, heavy metals may reduce species composition and limit microbial reproduction. Heavy metals have also been shown to affect microbial activity, such as nitrogen fixation in rhizobia [3,5]. Each heavy metal has unique biofunctions or biotoxicities. For example, copper can enhance microbial growth at low concentrations but suppress growth at high concentrations [6]. In contrast, cadmium has high toxicity at low concentrations.

Bioremediation is the most efficient and least costly method for treating heavy metal contaminated soils. Some reports have shown that indigenous microbes and plant–microbe symbionts tolerate high heavy metal concentrations in different ways and may play a significant role in the restoration of contaminated soil [7–9].

In this study, 108 bacterial strains were isolated from root nodules collected in tailings from the Taibai gold mining region in Shaanxi Province of China. The area, which came out of production 10 years ago, was badly contaminated by mining practices. The objective of this study was to isolate the most heavy metal resistant bacterial strain by comparing the growth rates and heavy metal bioaccumulation ability of bacterial strains collected at different periods and from different sites. The presence of resistance genes in the strain was confirmed by PCR analysis. In addition, physiological and biochemical features were used to characterize the strain, and phylogenetic analysis, based on 16S rRNA gene sequence data, was used to reveal the genetic relationships of the strain with other rhizobia.

2. Materials and methods

2.1. Isolation of strains and culture conditions

Root nodules of eight legume plant species, *Astragalus chrysopterus*, *Campylotropis macrocarpa*, *Indigofera pseudotinctoria*, *Lespedeza cuneata*, *Medicago lupulina*, *Pueraria lobata*, *Vicia cracce* and *Vicia unijuga*, were collected from the rhizosphere soil in tailings from the Taibai gold mine region of Shaanxi Province in northwest of China. We used yeast-mannitol agar medium (YMA) (3 g yeast extract, 10 g mannitol, 0.25 g K_2HPO_4, 0.25 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, and 20 g agar per liter) to isolate 108 strains of bacteria [10]. Single colonies were selected

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and checked for purity by repeated streaking and microscopic examination. All isolates were incubated at 28 °C and maintained either on YMA slants at 4 °C or in 20% (v/v) glycerol solution at −70 °C.

2.2. Screening test for heavy metal resistant strains

To isolate heavy metal resistant strains, the samples were screened on YMA plates, supplemented with four types of heavy metals at the following concentrations: $CuCl_2$, 0.1, 0.2, 0.3, 0.4 mM; $CdCl_2$, 0.1, 0.2, 0.3 mM; $Pb(NO_3)_2$, 0.05, 0.1, 0.5 mM; $ZnCl_2$, 1.0, 1.2, 1.4, 1.6 mM. We used a multi-point inoculator to add a 200-μl aliquot of cell suspension from each sample to the YMA plates and then incubated the cultures at 28 °C for 3–5 d. In order to confirm the results of the screening test, the strains tested above were inoculated into 5 ml TY liquid medium (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2{\cdot}2H_2O$ per liter), supplemented with four heavy metals at the following concentrations: Cu^{2+}, 0.3, 0.4, 0.5, 1.0, 2.0 mM; Cd^{2+}, 0.2, 0.3, 0.4, 1.0, 2.0 mM; Pb^{2+}, 0.3, 0.4, 0.5, 1.5, 2.0 mM; Zn^{2+}, 1.0, 1.6, 1.8, 2.0, 3.0 mM. The cultures were incubated at 28 °C and agitation at 150 rpm for 3–5 d. The growth values of the strains were determined by absorbance at 600 nm (OD_{600}). All tests were done in triplicate.

2.3. Determination of optimal heavy metal resistant strain

The specific growth rates of CCNWRS33-2 and CCNWRS08-1, which performed well in screening tests, were compared to determine which strain had the greatest resistance to heavy metals. Each strain was inoculated into TY medium with an initial density of 0.08 (OD_{600}) containing 2.0 mM Cu^{2+}, Cd^{2+}, Pb^{2+} or Zn^{2+} and then incubated at 28 °C and agitation at 150 rpm. Growth mass was determined by OD_{600} at 4 h intervals and the specific growth rate (μ) was obtained using the following formula [11].

$$\mu = \frac{1}{\mathrm{OD}_0} \times \frac{(\mathrm{OD}_t - \mathrm{OD}_0)}{(T_t - T_0)} \quad (1)$$

where $\mu\ h^{-1}$ denotes the specific growth rate of the initial bacteria concentration within a certain period of time; OD_t and OD_0 represent the optical density (600 nm) of the cultures at time t and 0; and T_t and T_0 represent corresponding times (h). The specific growth rate of each strain was determined at different times for each type of media, and the mean specific growth rate was calculated. The strain with the maximum mean specific growth rate was regarded as the optimal strain.

2.4. Effect of the presence of heavy metal on the growth of CCNWRS33-2

Stationary-phase cells of CCNWRS33-2 were inoculated into TY medium supplemented with different heavy metal ions. The initial density of the cell suspension was 0.08 (OD_{600}). The cultures were incubated for 24 h at 28 °C and agitation at 150 rpm. The concentrations of heavy metal in the TY medium were: Cu^{2+}, 1.0, 2.0 mM; Cd^{2+}, 0.5, 1.0, 2.0 mM; Pb^{2+}, 0.5, 1.5, 2.0 mM; Zn^{2+}, 1.0, 2.0, 3.0 mM. Growth rates of the strain were checked every 4 h by absorbance at 600 nm (OD_{600}) with a PerkinElmer UV/VIS spectrophotometer.

2.5. Characteristics of bioaccumulation of heavy metal in CCNWRS33-2 cells

An aliquot containing 0.25 g of cells (dry weight) was inoculated into TY medium containing 2.0 mM Cu^{2+}, 2.0 mM Cd^{2+}, 2.0 mM Pb^{2+} or 3.0 mM Zn^{2+} and incubated at 28 °C and agitation at 150 rpm. Cell pellets were 4, 12, 20, and 36 h after inoculation by centrifugation at 5000 rpm for 20 min. The pellets were washed with sterilized distilled water three times to remove free heavy metal ions. The cell pellets were treated with 10 mM sterilized EDTA at 28 °C and agitation for 30 min. The solution was centrifuged and then the supernatant and cell pellets were separated and analyzed with an atomic spectrophotometer to determine the heavy metal content in the cell walls and intracellular space.

2.6. Effect of different inoculation amounts on CCNWRS33-2 biomass under cadmium stress

Cadmium was chosen for the representative heavy metal in this test. An aliquot containing 0.05, 0.1, or 0.25 g (dry weight) of CCNWRS33-2 cells was inoculated into TY medium containing 0.5 mM, 1.5 mM, or 2.0 mM Cd^{2+}. The cultures were incubated at 28 °C and agitation at 150 rpm. Growth rate was determined by measuring absorbance at 600 nm (OD_{600}) at 4 h intervals.

2.7. Amplification of copA partial sequence and 16S rDNA

The strain CCNWRS33-2 was incubated in TY medium, and the total DNA was extracted using the method reported by Terefework et al. [12].

Primers for the amplification of a Cu-resistance gene (*copA*) were *copA*1: 5′-TGCAACAGAACGGCACCTAy (T/C)TGGTr (G/A)b (C/G/T)CA-3′ (forward) and *copA*2: 5′-CGGGCGAAACAGGCCn (G/C/A/T)GTCCAr(G/A) TT-3′ (reverse) [9]. Pb-resistant gene (*pbrA*) primers were *pbrA*1: 5′-ATG AGC GAA TGT GGC TCG AAG-3′ (forward) and *pbrA*2: 5′-TCATCGACGC AACAGCCTCAA-3′ (reverse) [13]. Cd-resistant gene (*czcA*) primers were *czcA*1: 5′-GTTTGAACGTATCATTAGT TC-3′ (forward) and *czcA*2: 5′-GTA GCC ATC CGA AAT ATT CG-3′ (reverse) [14]. Zn-resistant gene (*czcD*) primers were *czcD*1: 5′-CAG GTC ACT GAC ACG ACC AT-3′ and *czcD*1: 5′-CAT GCT GAT GAG ATT GAT GAT C-3′ [14]. The conditions for PCR amplification were: pre-denaturalization at 95 °C for 3 min, then 30 cycles of denaturing at 94 °C for 1 min, annealing for 1 min (56 °C for *cop*A, 60 °C for *pbr*A and *czc*A, 50 °C for *czcD*), extension at 72 °C for 2 min, and a final step for extension at 72 °C for 10 min.

16S rDNA was amplified with primers P1 and P6 (P1: 5′-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3′; P6: 5′-TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3′). The PCR conditions used were: an initial denaturation step at 94 °C for 3 min; followed by 30 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 10 min [15].

PCR products were detected by electrophoresis on 1% TBE agarose gel, stained with ethidium bromide and visualized using a BioRad UV-transilluminator. PCR bands were excised from the gel and DNA was extracted from the agarose gel using HQ-20 PCR DNA and Gel Band Purification Kit from Anhui U-gene Biotechnology Co., Ltd. After purification, the PCR product was sequenced directly using an ABI PRISM 377 DNA Sequencer (PerkinElmer Applied Biosystems).

2.8. Homogenous analysis of CopA and phylogenic analysis of 16S rRNA gene

Protein sequences of CopA and the nucleotide sequence of 16S rRNA gene were aligned and a phylogenetic tree was constructed with ClustalX v.1.81 software using the neighbour-joining method. The genetic distance of relevant genes was calculated with Bioedit Software [16].

Table 1
Specific growth rate of resistant strain in different heavy metals stress ($\mu\ h^{-1}$)

Heavy metal	CCNWRS33-2	CCNWRS08-1
Cu^{2+}	0.62	0.220
Cd^{2+}	0.3	−0.054
Zn^{2+}	0.25	0.206
Pb^{2+}	−0.31	−0.25
Mean specific growth rate	0.21	0.02

2.9. Physiological and biochemical characteristics of CCNWRS33-2

Sixty-seven physiological and biochemical features, including utilization of sugars and organic acids as sole sources of carbon, utilization of amino acids as sole sources of nitrogen, resistance to antibiotics, dyes, and chemicals, tolerance of different NaCl concentrations, temperature and pH ranges for growth and litmus milk reaction, reduction of methylene blue and nitrate, production of urease and catalase, and acid or alkali production were tested [15]. All tests were done in triplicate.

3. Results

3.1. Screening of heavy metal resistant strains

Among the 108 bacterial strains, 15 strains grew in TY liquid medium supplemented with 0.2 mM Cu^{2+} and three strains grew in TY liquid medium supplemented with 2.0 mM Cu^{2+}. Four strains grew in TY liquid medium supplemented with 2.0 mM Cd^{2+}. Six strains grew in TY liquid medium supplemented with 2.0 mM Pb^{2+} and three strains grew in TY liquid medium containing 3.0 mM Zn^{2+}. Finally, two strains, CCNWRS33-2 and CCNWRS08-1, showed high resistance to copper, cadmium, lead and zinc. These two strains were used to determine the optimal heavy metal resistant strain.

The specific growth rates and the mean specific growth rates of the two strains were calculated using the formulas described previously (Table 1). According to the specific growth rates, the four heavy metals differed in their inhibitory effects on the strain. The ranking of the inhibitory effects of the four heavy metal ions on CCNWRS33-2 declined in the order $Pb^{2+} > Zn^{2+} > Cd^{2+} > Cu^{2+}$ compared to a ranking of $Pb^{2+} > Cd^{2+} > Zn^{2+} > Cu^{2+}$ for CCNWRS08-1. The mean specific growth rate of CCNWRS33-2, 0.21 $\mu\ h^{-1}$, was higher than the mean specific growth rate of CCNWRS08-1. Therefore, strain CCNWRS33-2 was determined to be the strain with maximum heavy metal resistance and it was used for further study.

3.2. Growth of CCNWRS33-2 in the presence of each heavy metal

The growth of strain CCNWRS33-2 was greater when the TY medium was supplemented with 1.0 mM or 2.0 mM Cu^{2+} than when there was no Cu^{2+} after 4 h incubation (Fig. 1A). After 8 h incubation, the growth of CCNWRS33-2 was 2.3 times greater in TY medium containing 1.0 mM Cu^{2+} compared to in TY medium without Cu. Similarly, the growth of CCNWRS33-2 in TY medium containing 2.0 mM $Cu^{2+}$was 1.3 times more than in TY medium without Cu after 24 h. There was no significant difference in the OD_{600} of cultures grown at 1.0 mM and 2.0 mM Cu^{2+} after 20–24 h incubation.

The growth of strain CCNWRS33-2 was significantly reduced when the TY medium contained Cd^{2+}, indicating that Cd^{2+} had a high degree of toxicity on the strain. After the 8 h lag phase, the growth of the strain continually declined until 12 h in TY medium containing Cd^{2+}. The biomass was 75% lower in TY medium containing 2.0 mM Cd^{2+} compared to in TY medium without Cd^{2+} after 24 h incubation (Fig. 1B).

The effect of Pb^{2+} on strain CCNWRS33-2 varied significantly depending on the Pb^{2+} concentration (Fig. 1C). Results showed that 0.5 mM Pb^{2+} had only a small affect on the growth of the strain. At higher concentrations (1.5 and 2.0 mM Pb^{2+}), the lag phase lasted for about 30 h. During the lag phase, the OD_{600} dropped to −0.23. After 30 h the growth rate increased rapidly and by 70 h there was no significant difference in the OD_{600} among the four treatments.

Growth curves for strain CCNWRS33-2 in the presence of Zn^{2+} are shown in Fig. 1D. The growth curve for TY medium containing

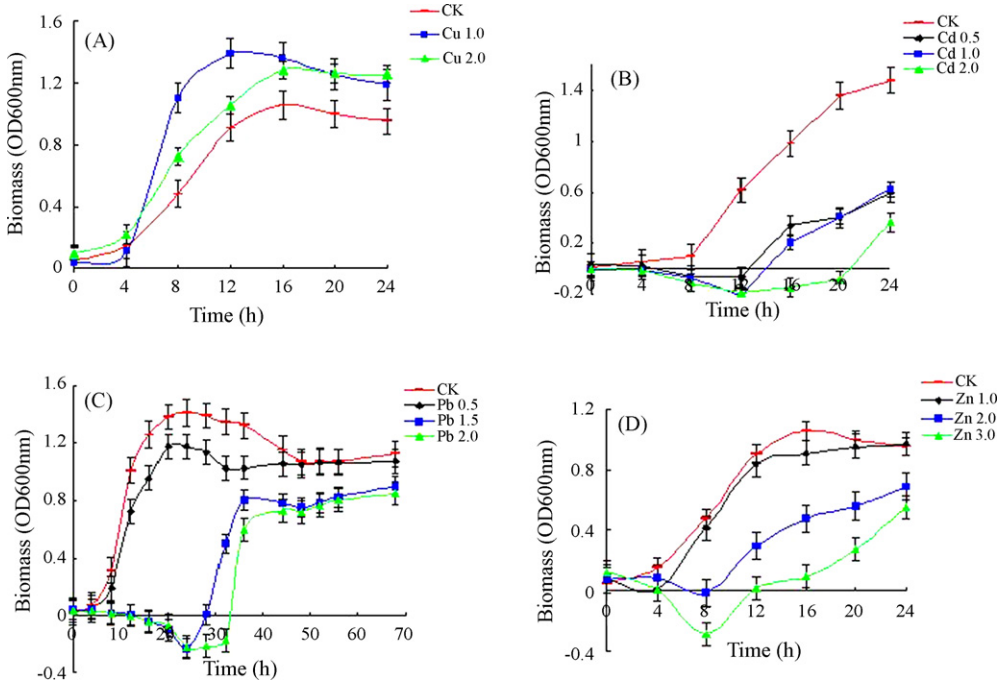

Fig. 1. Growth rates of CCNWRS33-2 in the presence of different heavy metals.

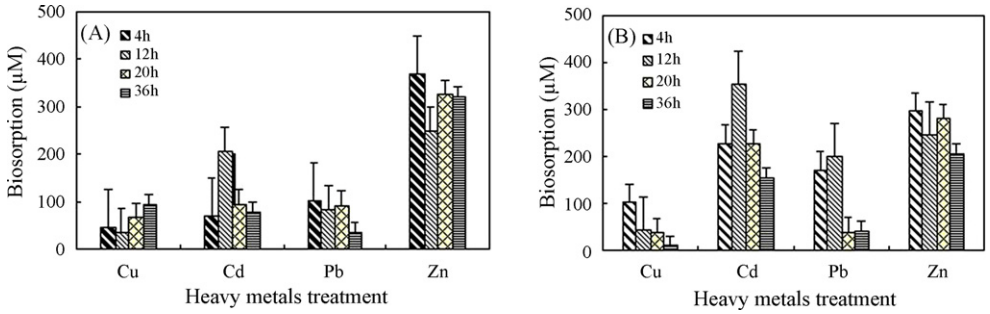

Fig. 2. Accumulation of heavy metals in CCNWRS33-2 (A, cell wall; B, intracellular space).

1.0 mM Zn^{2+} was similar to that of TY medium without Zn^{2+}, with ordinary lag and exponential phases. Growth curves for the strain in TY medium containing 2.0 or 3.0 mM Zn^{2+} displayed the same trends, but the OD_{600} value in TY medium containing 3.0 mM Zn^{2+} was lower than in TY medium containing 2.0 mM Zn^{2+}. The biomass of CCNWRS33-2 was 72% larger in the 2.0 mM Zn^{2+} treatment and 58% larger in the 3.0 mM Zn^{2+} treatment compared to the control (no Zn^{2+}) treatment.

3.3. Heavy metal bioaccumulation in CCNWRS33-2

A comparison of heavy metal accumulation in cell walls of CCNWRS33-2 is shown in Fig. 2A. The mean Cu^{2+} content in the cell walls of strain CCNWRS33-2 was 60 μM, the lowest amount among the four heavy metals tested in this study. Generally, moderate amounts of Cd^{2+} and Pb^{2+} accumulated in cell walls. The exception was that a relatively large amount (205 μM) of Cd^{2+} was measured in cells walls at 12 h. After 36 h incubation, the amount of Pb^{2+} in cell walls dropped to 36 μM. Mean accumulation of Zn^{2+} in the cell walls was 316 μM, the largest amount among the four heavy metals tested in this study.

Measurements of intracellular accumulation indicated that Cu^{2+} was the least absorbable heavy metal (Fig. 2B). After 4 h incubation, intracellular accumulation of Cu^{2+} was 101 μM, which was nine times more than it was at 36 h. Intracellular accumulation of Cd^{2+} increased from 225 μM at 4 h to a peak of 353 μM at 12 h. For Pb^{2+}, intracellular accumulation reached 200 μM at 12 h and then dropped sharply dropped to 38 μM from 20 to 36 h. Similar to our observations for accumulation in cell walls, intracellular accumulation was greatest for Zn^{2+} with a mean bioaccumulation amount of 257 μM.

3.4. Effect of inoculation amount on CCNWRS33-2 under Cd stress

When strain CCNWRS33-2 was incubated under Cd^{2+} stress, biomass generally increased as inoculation size increased (Fig. 3). After 96 h incubation in the presence of 2.0 mM Cd^{2+}, biomass in the treatment inoculated with 0.25 g of cells was three times more than the biomass in the treatments which were inoculated with 0.05 or 0.10 g. The growth curve for the strain showed evidence of secondary growth. The growth rate increased at the beginning of the incubation then reached a plateau phase. Later, the growth rate

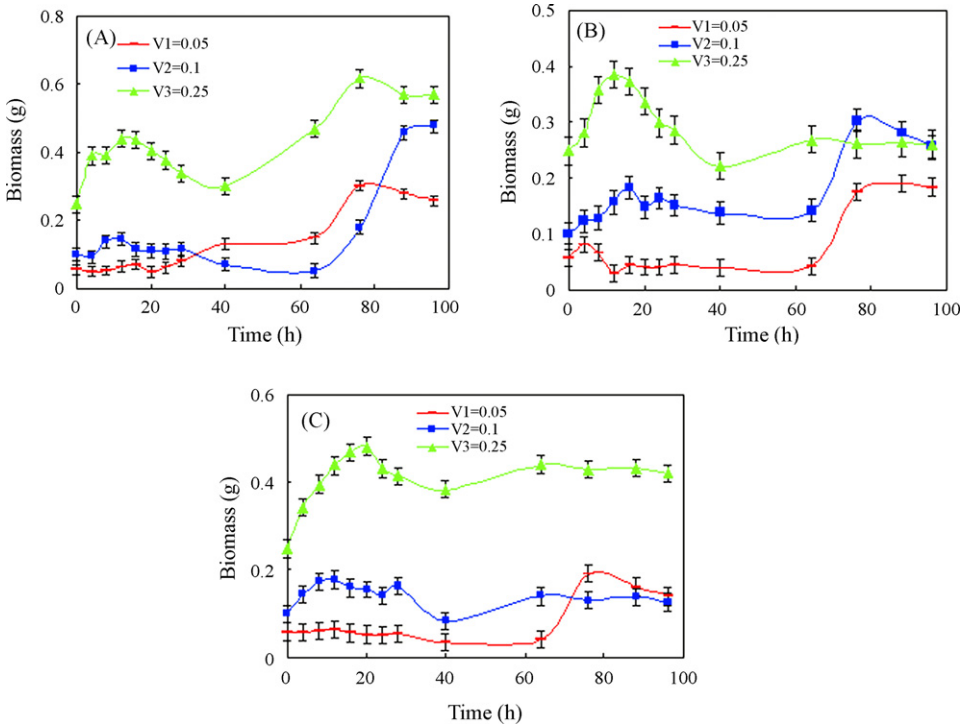

Fig. 3. Effect of inoculation amount on CCNWRS33-2 under Cd stress (A, 0.5 mM Cd^{2+}; B, 1.5 mM Cd^{2+}; C, 2.0 mM Cd^{2+}).

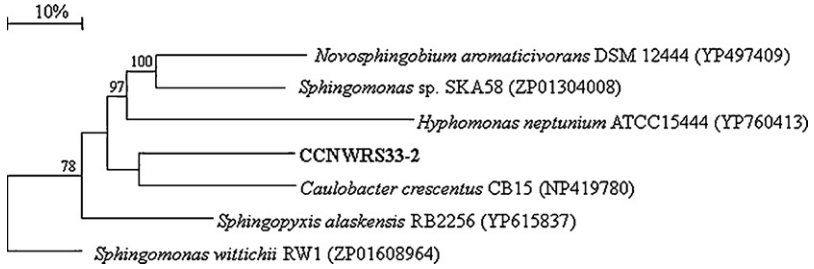

Fig. 4. Phylogenetic tree constructed based on copper resistance protein CopA by neighbour-joining method. Numbers at nodes indicate bootstrap values. 10% denotes the genetic distance.

began to increase again. Secondary growth was most significant in the treatment which had low Cd^{2+} in the TY medium and high inoculation amount.

3.5. CopA homologous analysis

The primers used for the amplification of the Cu-resistant gene *cop*A yielded a band of approximately 360 bp. The nucleotide sequence was translated into a protein sequence and aligned with CopA protein partial sequences of other Cu-resistant bacteria. The phylogenetic tree based on the CopA protein partial sequence was constructed by Treecon W and similarities were generated (Fig. 4). Sequence comparisons with our Cu-resistant PCR fragment showed a high homology with Cu-resistant genes from other bacteria (70.1% with a Cu-resistant protein from *Caulobacter crescentus* CB15 [NP419780], 69% with CopA from *Sphingopyxis alaskensis* RB2256 [YP615837] and 66.6% with the CopA from *Sphingomonas* sp. SKA58 [ZP01304008]).

3.6. 16S rDNA phylogenic analysis

Sequence analysis of the 16S rRNA gene is a fast and accurate method to identify the phylogenic position of bacteria. Full-length (about 1500 bp) 16S rDNA of CCNWRS33-2 was sequenced and used to construct a phylogenetic tree (Fig. 5). We found that strain CCNWRS33-2 was classified in the branch of *Rhizobium–Agrobacterium* genera. It had 98.9% similarity with *A. tumefaciens* LMG196 (X67223) and 95.9% similarity with *R. giardinii* H152 (U86344).

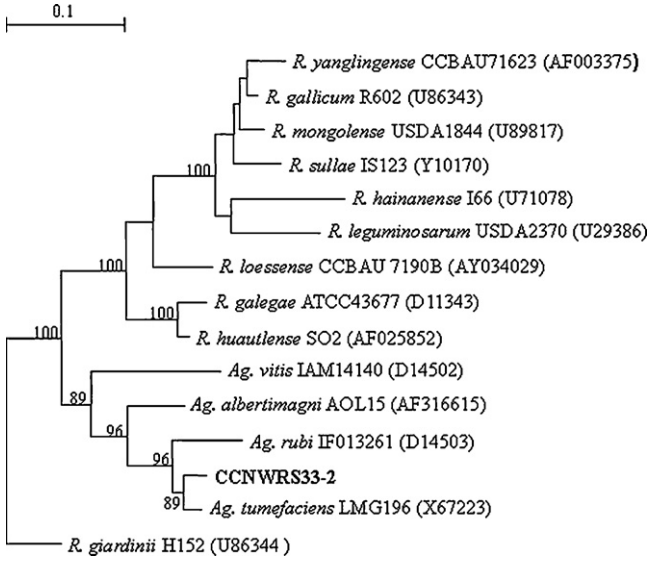

Fig. 5. Phylogenetic tree constructed based on 16S rDNA of CCNWRS33-2 and reference strains by the neighbour-joining method. Numbers at nodes indicate bootstrap values. 0.1 denotes the genetic distance.

3.7. Physiological and biochemical characteristics of CCNWRS33-2

The physiological and biochemical characteristics of strain CCNWRS33-2 were shown in Table 2. The strain had a wide pH tolerance of 9.0–11.0, and salt tolerance was up to 5% NaCl. CCNWRS33-2 could use the following materials as sole carbon sources: sucrose, sodium hippurate, sodium citric acid, erythritol, arabinose, rhamnose, glucogen, galactose, D-xylose, sodium pyruvic acid, amylomaltose, galactosylglucose, and D-fructose. Alanine, L-norleucine, L-leucine, L-aspartate, L-cysteine, glycine, DL-histidine, L-glutamate and L-tryptophan could be used as sole nitrogen sources. CCNWRS33-2 was resistant to the following antibiotics: penicillin (5 μg/ml), streptomycin (5–50 μg/ml), fosfomycin (5–300 μg/ml), lincomycin (5–300 μg/ml) and chloromycetin (5–300 μg/ml). It could grow in a medium supplemented with neutral red (0.2%), methylene blue (0.2%), congo red (0.1%), methyl red (0.2%), methyl orange (0.2%) and thymol blue (0.2%). CCNWRS33-2 produced acid and catalase in YMA and was a nitrate-reducer.

4. Discussion

4.1. Identification of the strain with optimal resistance and its growth in the presence of heavy metals

The root nodule samples were collected at mine tailing region which has a history of pollution from mine waste. From the 108 strains, CCNWRS33-2 and CCNWRS08-1 exhibited high resistance to Cu, Cd, Pb and Zn. The mean specific growth rate of strain CCNWRS33-2 in the presence of 2.0 mM Cu^{2+}, Cd^{2+}, Pb^{2+} or Zn^{2+} was greater than the mean specific growth rate for CCNWRS08-1. Therefore, we selected CCNWRS33-2 as the strain with optimal resistance.

Measurement of the growth rate of CCNWRS33-2 in the presence of Cd, Cu, Pb, or Zn indicated differences in toxicity to the bacteria among the heavy metals. Specifically, 0.5–2.0 mM Cd^{2+} inhibited the growth of CCNWRS33-2 significantly. The effects of Zn and Pb were similar. At low concentrations (0.5 mM Pb^{2+} and 1.0 mMZn^{2+}), the two heavy metals had little influence on CCNWRS33-2, but at high concentrations ($\geq$1.5 mM Pb^{2+}, $\geq$2.0 mM Zn^{2+}) the two heavy metals prolonged the lag phase and reduced the maximum biomass. Our observation that 2.0 mM Cu^{2+} increased the growth rate of CCNWRS33-2 was different from other reports that Cu^{2+} resulted in a decrease in the growth rate of resistant bacterial strains [1,5,9]. It has been reported that Cu increased the growth of microbes at 10 mg/l (0.157 mM), but tended

Table 2
The physiological and biochemical characteristics of CCNWRS33-2

Physiological characteristics		Antibiotics and dyes resistance		Sole carbon and nitrogen sources (1 mg/ml)		Biochemical characteristics	
Growth at temperature		Penicillin (5 μg/ml)	+	Sucrose	+	Litmus milk alkali production	–
4 °C	–	Penicillin (50, 100, 300 μg/ml)	–	Sodium hippurate	+	Litmus milk acid production	+
10 °C	–	Streptomycin (5, 50 μg/ml)	+	Sodium citric acid	+	Litmus milk peptonization	–
28 °C	+	Streptomycin (100, 300 μg/ml)	–	Erythritol	+	Litmus milk reduction	–
40 °C	–	Fosfomycin (5, 50, 100, 300 μg/ml)	+	Arabinose	+	Reduction of methylene blue	–
60 °C	–	Lincomycin (5, 50, 100, 300 μg/ml)	+	Sorbose	–	Reduction of nitrate	+
Growth at pH		Chloromycetin (5, 50, 100, 300 μg/ml)	+	Rhamnose	+	Production of urease	–
4	–	Neutral red (0.1, 0.2%)	+	Glucogen	+	Production of catalase	+
5	–	Methylene blue (0.1, 0.2%)	+	Galactose	+	Acid production	+
9	+	Congo red (0.1%)	+	D-Xylose	+	Alkali production	–
10	+	Methyl green (0.1, 0.2%)	–	Sodium pyruvic acid	+		
11	+	Methyl red (0.1, 0.2%)	+	Amylomaltose	+		
Growth on NaCl (%)		Methyl orange (0.1, 0.2%)	+	Galactosylglucose	+		
1	+	Thymol blue (0.1, 0.2%)	+	D-Fructose	+		
2	+			Alanine	+		
3	+			L-Norleucine	+		
4	+			L-Leucine	+		
5	+			L-Tyrosine	–		
				L-Aspartate	+		
				L-Cysteine	+		
				Glycine	+		
				L-Glutamate	+		
				L-Tryptophan	+		
				DL-Histidine	+		

to decrease the growth rate at >50 mg/l (0.787 mM). All of these concentrations were significantly lower than the concentration of 2.0 mM that was tested in our study.

4.2. Accumulation of heavy metals

The accumulation of Zn in the cell walls and intracellular space of strain CCNWRS33-2 was greater than the accumulation of the other three heavy metals tested in this study. Intracellular accumulation of Cd and Pb was greater than accumulation in the cell walls. Accumulated amounts of Cu, Cd, and Pb were greatest after 4–12 h incubation and then gradually declined After 36 h incubation, the amount of Cu was reduced to 70.1% of its maximum, while the amounts of Cd and Pb were at 41.4 and 27.2% of their maximum values. This suggests extrusion as a possible mechanism of heavy metal resistance in CCNWRS33-2.

4.3. Amplification of heavy metal resistant gene

Through the evolutionary process, microbes have been improving their heavy metal resistance mechanisms to adapt to adverse environments. Products encoded by heavy metal resistant genes can reduce or eliminate heavy metal toxicity. Some heavy metal resistant gene primers have been selected for PCR amplification and sequence analysis. PCR amplification and electrophoresis showed that CCNWRS33-2 contained only the gene *copA,* but other heavy metal resistant genes, including *pbrA* and *czcA* were not found. It is possible that the primers used to amplify *pbrA* and *czcA* in this study were inappropriate. We need to investigate this further. It may have no relationship with the Pb, Cd and Zn resistance mechanisms that the strain use *pbr* and *czc* operon. There may be other heavy metal resistance (Pb, Cd and Zn) systems. Rosen found that efflux protein gene coding efflux protein to accelerate Cd^{2+} discharge also lies in the chromosome [17]. Llanos reported that some resistant-cadmium strains lacked plasmids [18]. They showed that the cadmium resistance gene was not always controlled by plasmids, but may be controlled by chromosomes [19].

The sequence comparison with the Cu-resistant PCR fragment showed a high homology of the Cu-resistant genes with other bacteria. The nucleotide sequence was translated into protein sequences and aligned with the CopA protein partial sequence of other Cu-resistant bacteria. The CopA phylogenetic tree analysis showed that CCNWRS33-2 formed a phylogenetic branch with *C. crescentus*. The *cop* operon, which mainly includes four open reading frames (ORFs) *copA*, *copB*, *copC* and *copD*, is a kind of copper resistant operon. *CopA* and *copB* are essential genes for copper resistance. Their expressed proteins, located in different cell positions, play different roles in the bacteria. CopA, located in the periplasmic space, catalyzes the intake of copper while CopB, located in the outer membrane, catalyzes the efflux of copper. CopC, just like CopA, is located in the periplasmic space and CopD is located in the inner membrane. It has been stated that resistant genes played a role in rhizobial resistance to heavy metals. The reason why rhizobia contained heavy metal genes may be from gene level transfer [20,21].

5. Conclusions

The strain CCNWRS33-2 isolated from the root nodule of *L. cuneata* in gold mine tailings showed significant heavy metal resistance. Our observations for heavy metal accumulation within the cell and cell wall combined with other research reports suggest that extrusion from the intracellular space is one possible mechanism for heavy metal resistance in this strain. The existence of the *cop*A gene was determined by PCR, and it showed high homology with Cu-resistant genes from other bacteria. Based on 16S rDNA sequence analysis, strain CCNWRS33-2 belongs to the *Rhizobium–Agrobacterium* branch.

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