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Characterization of Copper-Resistant *Agrobacterium* Isolated from Legume Nodule in Mining Tailings

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Abstract A copper-resistant bacteria CCNWSX2332 was isolated from root nodules of *Lespedeza cuneata* growing in a gold mining tailing region in northwest of China. The specific growth rate of the strain was 0.62 μh^{-1} in the presence of 2.0 mM Cu^{2+} in TY liquid media, and the maximum copper accumulation of whole cell reached 147.03 μM Cu^{2+} per gram (dry weight) after 4 h incubation. A partial sequence of the copper resistance gene *cop*A was amplified from the strain, and the phylogenetic analysis based on 16S rDNA sequence showed that CCNWSX2332 belonged to *Agrobacterium*, and it had 100% similarity with *Agrobacterium tumefaciens* type strain IAM13129^T.

Keywords *Agrobacterium* · Copper · *Cop*A gene · Phylogeny

Heavy-metal contamination is now a pressing issue, many agricultural and industrial practices have led to environmental pollution by heavy metal ions. It has caused an evident effect on indigenous microbial population, impoverishing their diversity and influencing some special activities such as nitrogen-fixation in rhizobia (Chaudri et al. 2000). Copper, as our focus in this study, is different from other heavy metals such as cadmium or lead. It is an essential trace element, which will enhance the growth of microbes at very low concentrations. At high concentrations, however, it will also harm microbes in various aspects (Ledin 2000; Karnachuk et al. 2003). Among the methods available to deal with copper contamination, bioremediation is a relatively efficient and low cost way. Some studies have shown that plant-microbe symbiosis may play a significant role in restoration of polluted soil (Delorme et al. 2003; Carrasco et al. 2005).

In this study, copper-resistant bacteria were isolated from root nodules of *Lespedeza cuneata* growing in the Taibai gold mining tailing region. Characteristic of copper resistance was determined, including growth in the presence of copper and copper accumulation in different sites and periods. The copper resistant gene was amplified to confirm the resistant mechanism. In addition, phylogenetic analysis based on 16S rRNA gene was used to reveal the genetic relationship of the strain.

Materials and Methods

Bacteria were isolated from nodules of *Lespedeza cuneata*, which grew in the Taibai gold mining tailing of Shaanxi province in northwest of China, with a standard method and by yeast-mannitol agar medium (YMA) (Vincent 1970). 19 isolates were obtained after purity identification. All isolates were incubated at 28°C and maintained on YMA slants at 4°C, or in 20% (v/v) glycerol at −80°C.

The isolates were primarily screened by YMA supplemented with 0, 0.2, 0.3, 0.4 mM $CuCl_2$, respectively. A 200 μl cell suspension of each isolate was inoculated by a multi-point inoculator, then incubated at 28°C for 3–5 days. The strains growing in a primary screening test were inoculated into 5 mL TY liquid medium supplemented with 0, 0.3, 0.4, 0.5, 1.0, 2.0 mM Cu^{2+}, respectively, and were incubated at 28°C with shaking at 150 rpm for 3–5 d.

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The growth of strains was determined by absorbance at 600 nm (OD_{600}) with a Perkin-Elmer UV/VIS spectrophotometer. All tests were done in triplicates and no additional copper was used as a control.

The tested strain was inoculated into TY medium containing 2.0 mM Cu^{2+} with an initial density of 0.08 (OD_{600}). The growth mass was determined by OD_{600} for 4 h intervals and the specific growth rate (μ) was obtained using the formula (1).

$$\mu = 1/OD_0 \times (OD_t - OD_0)/(T_t - T_0) \quad (1)$$

Stationary-phase cells of CCNWSX2332 with an initial density of 0.08 (OD_{600}) were inoculated into TY medium supplemented with 0, 1.0, 2.0 mM Cu^{2+}, respectively, and were incubated at 28°C with shaking at 150 rpm for 24 h.

The equivalent of 0.25 g cell body (dry weight) was inoculated into TY media containing 2.0 mM Cu^{2+} at 28°C and agitation at 150 rpm. The pellet was harvested by centrifugation at 5,000 rpm for 20 min after 4, 12, 20 and 36 h inoculation, respectively, and washed by sterilized distilled water for 3 times to remove free Cu^{2+}. Then, the pellet was treated by 10 mM sterilized EDTA at 28°C, shaking for 30 min. Supernatant and sediment were collected by centrifugation, respectively. Cu^{2+} concentration distributed in these two fractions was determined by atomic-spectrophotometer methods. The concentration of Cu^{2+} in supernatant was considered as the accumulation of wall-bound and in sediment as intracellular accumulation (Carrasco et al. 2005).

The strain CCNWSX2332 was incubated in TY media, and total DNA was extracted following Terefework et al. (1998). A partial *cop*A gene (about 360 bp fragment) was amplified with the primers [*cop*A1: 5′-TGC AAC AGA ACG GCACCT Ay(T/C)T GGT r(G/A)b (C/G/T)C A-3′; *cop*A2: 5′-CGG GCG AAA CAG GCC n(G/C/A/T)GT CCA r(G/A) TT-3′]. The conditions for PCR amplification were as follows: an initial denaturalization step of 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 56°C for 75 s, 72°C for 2 min and a final extension step at 72°C for10 min (Carrasco et al. 2005). The 16S rDNA was amplified using the method and primers reported by Liu et al. (2005).

Results and Discussion

Among the 19 strains, three strains CCNWSX2332, CCNWSX2172 and CCNWSX2442 could grow in TY liquid medium supplemented with 0.2 mM Cu^{2+}, and CCNWSX2332 could grow in TY liquid media supplemented with 2.0 mM Cu^{2+}. The specific growth rate of CCNWSX2332 was 0.62 μh^{-1} in presence of 2.0 mM Cu^{2+}.

The growth of CCNWSX2332 in TY media supplemented 1.0 mM and 2.0 mM Cu^{2+} was greater than that without Cu^{2+} after 4 h incubation (Fig. 1). The growth of CCNWSX2332 in the presence of 1.0 mM Cu^{2+} was 2.3 times greater than that in TY media without Cu after 8 h incubation. Similarly, the growth of CCNWSX2332 in TY media containing 2.0 mM Cu^{2+} was 1.3 times more than that in TY media without Cu after 24 h. Density of the culture in the presence of 1.0 mM or 2.0 mM Cu^{2+} after 20–24 h incubation showed not significant differences.

The highest copper accumulation appeared in whole cells at 4 h, reaching 147.03 μM Cu^{2+} per gram (dry weight) (Fig. 2). The copper accumulation also varied between different cell sites and incubation times. The intracellular copper accumulation gradually declined from 4 h to 36 h, when the accumulation of copper at 4 h was 9 times more than it at 36 h. Copper accumulation in cell walls varied,

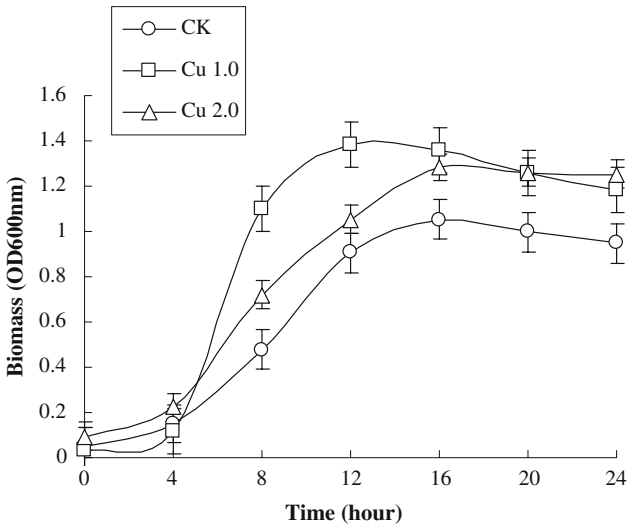

Fig. 1 The growth of strain CCNWSX2332 in different Cu^{2+} concentrations

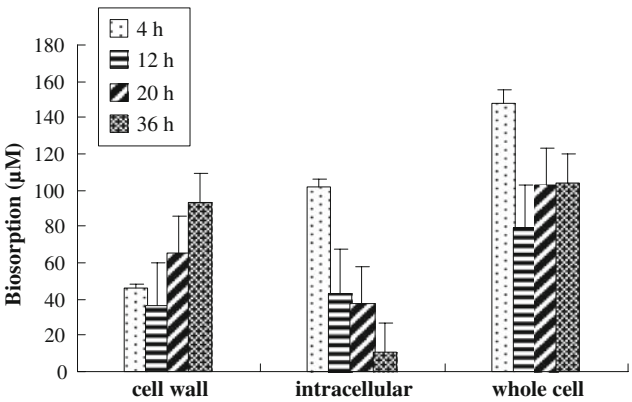

Fig. 2 Copper accumulation of strain CCNWSX2332 at different sites and incubation times

with the order of copper accumulation being 36 h > 20 h > 4 h > 12 h. This phenomenon suggested that copper was extruded from cell and bound in cell wall. A possible mechanism of copper resistance in CCNWSX2332 was extrusion.

An approximately 360 bp fragment was amplified using the primers of Cu-resistant gene *cop*A and sequenced (Fig. 3). The nucleotide sequence was translated into a protein sequence and aligned with CopA protein partial sequence of other Cu-resistant bacteria. The similarity based on CopA protein partial sequence was calculated using BioEdit software (Table 1). The CopA protein partial sequence of CCNWSX2332 had a relatively high homology with Cu resistant genes from other bacteria. The similarity with *Caulobacter crescentu* CB15 and *Sphingopyxis alaskensis* RB2256 was 70.7%. Existence of partial sequence of *cop*A, one in four ORFs of *cop* operon, suggested a possible resistant gene source.

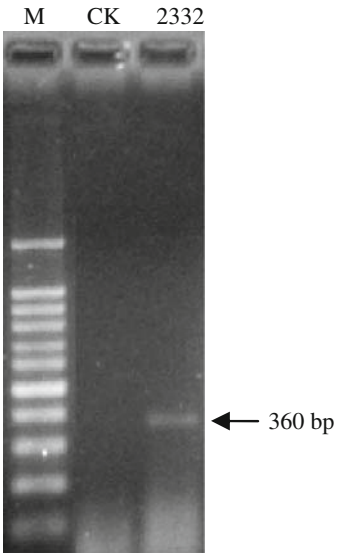

Fig. 3 PCR amplification of fragments of Cu resistance genes

About 1.5 kb fragment of 16S rDNA was amplified and sequenced. GenBank Blast searches indicated that the strain CCNWSX2332 belonged to *Agrobacterium* genera. A more precise taxonomic position was then assessed by phylogenetic comparisons with reference strains. The phylogenetic tree indicated that CCNWSX2332 was identical to that of the *Agrobacterium tumefaciens* type strain IAM13129^T with 100% similarity (Fig. 4).

The nodule samples were collected at a mining tailings region which has a history of pollution by mine wastes. Three strains of 19 isolates had resistances to copper at 0.2 mM, and one strain could grow in presence of 2.0 mM Cu^{2+}. The level of copper resistance in the isolates was comparable to that of other bacteria from different genera (Yilmaz 2003; Carrasco et al. 2005; Figueura et al. 2005). Behavior of CCNWSX2332 in the presence of copper was

Table 1 Similarity (%) of CopA protein partial sequence of CCNWSX2332 to reference strains

Strain	CCNWSX2332	CB15	RB2256	DSM1244	RW1
CCNWSX2332	100	70.7	70.7	65.0	65.0
Caulobacter crescentus CB15 (NP_419780)	70.7	100	71.6	62.2	70.7
Sphingopyxis alaskensis RB2256 (YP_615837)	70.7	71.6	100	61.3	77.3
Novospingobium aromaticivorans DSM1244 (YP_497409)	65.0	62.2	61.3	100	64.1
Sphingomonas wittichii RW1 (YP_001262718)	65.0	70.7	77.3	64.1	100

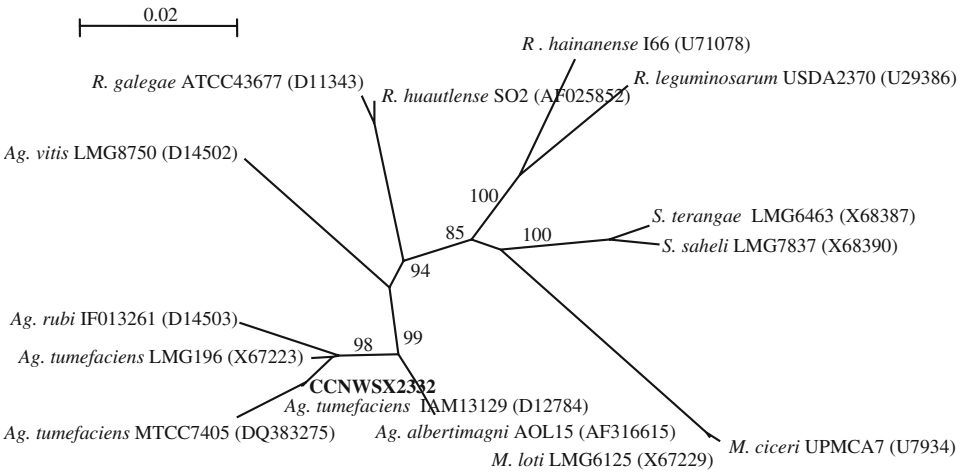

Fig. 4 Unrooted phylogenetic trees of CCNWSX2332 (shown in bold) and reference strains based on neighbor-joining analysis. Bootstrap percentages above 75% are indicated, and 0.02 denotes genetic distance. Ag: *Agrobacterium*; M: *Mesorhizobium*; R: *Rhizobium*; S: *Sinorhizobium*

peculiar. The growth of the strain was enhanced by concentrations up to 2.0 mM Cu^{2+}, which was not similar to the situation of other resistant strains whose growth declined in the presence of copper (Adriaensen et al. 2005). Although it is reported that copper could enhance the growth of microbe at 10 mg/L (0.157 mM), and tends to decrease the growth rate at 50 mg/L (0.787 mM), which was evidently lower than the concentration of 2.0 mM that was tested in our study.

Further study to reveal the mechanism of copper resistance in the strain, as well as the function of *cop*A, is ongoing.

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