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Characterization of a copper-resistant symbiotic bacterium isolated from *Medicago lupulina* growing in mine tailings

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ABSTRACT

A root nodule bacterium, *Sinorhizobium meliloti* CCNWSX0020, resistant to 1.4 mM Cu^{2+} was isolated from *Medicago lupulina* growing in mine tailings. In medium supplied with copper, this bacterium showed cell deformation and aggregation due to precipitation of copper on the cell surface. Genes similar to the copper-resistant genes, *pcoR* and *pcoA* from *Escherichia coli*, were amplified by PCR from a 1.4-Mb megaplasmid. Inoculation with *S. meliloti* CCNWSX0020 increased the biomass of *M. lupulina* grown in medium added 0 and 100 mg Cu^{2+} kg^{-1} by 45.8% and 78.2%, respectively, and increased the copper concentration inside the plant tissues grown in medium supplied with 100 μM Cu^{2+} by 39.3%, demonstrating that it is a prospective symbiotic system for bioremediation purposes.

1. Introduction

Copper is an essential trace element, serving as a cofactor for a variety of enzymes, such as ascorbic acid oxidase, polyphenol oxidase and superoxide dismutase, and electron transfer proteins involved in redox reactions (Brown et al., 1997). However, excess copper damages cells by metal-catalyzed oxidation of proteins and by causing oxidative damage through the generation of reactive oxygen species (ROS) (Lebedev et al., 2002). At minimal or excessive metal levels, the uptake systems in a microorganism are important factors for survival and competitiveness (Silver and Walderhaug, 1992). Generally, microbial cells have evolved effective and accurate mechanisms to maintain copper homeostasis and control copper levels by converting free Cu^{2+} to nontoxic organic copper complexes (Rensing and Grass, 2003). Copper homeostasis is a complicated process involving copper sequestration, uptake and efflux (Rensing and Grass, 2003). The genes related to these mechanisms may be encoded on the chromosome, but loci conferring resistance are usually located on plasmids (Rensing and Grass, 2003). Two different plasmid-encoded copper resistance systems *pco* and *cop* have been described in bacteria. The *pco* system, involving energy-dependent uptake and efflux of copper ions, has been expounded in *Escherichia coli* (Rouch et al., 1985), and a chromosome-encoded copper uptake and intracellular transportation system is also connected with the *pco* system in this bacterium (Brown et al., 1994). Another energy-dependent uptake and efflux process for copper (cop system) that is accompanied by sequestration of copper in the periplasm and outer membrane has been described in *Pseudomonas syringae* (Cooksey, 1994).

The development of mining, smelting and processing of copper, has caused contamination of some soils with this metal in China (Chen et al., 1999), and bioremediation might be helpful for restoring such soils (Wu et al., 2010). Some microorganisms and plant–microbe symbiosis can tolerate high concentrations of heavy metals and may be useful for the restoration of contaminated soils (Carrasco et al., 2005). For example, inoculation of pea grown in metal-amended soil with a nickel and zinc-resistant strain, *Rhizobium* sp. RP5, isolated from pea nodule increased dry matter and yield of seeds, while decreasing the concentration of nickel and zinc in the plants (Wani et al., 2008).

In order to develop a collaborative restoration system of microorganisms and plants in copper-contaminated soils from mine tailings, legumes grown in the mine tailings were surveyed and effective copper-resistant microsymbionts were isolated from their root nodules. The tolerance level, resistance mechanisms and resistance-related genes of this isolate were investigated.

2. Methods

2.1. Isolation and screening of copper-resistant rhizobia

Root nodules were collected from *Medicago lupulina* growing in the heavy metal mine tailings of Fengxian county, China

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(33°36′29″ N, 107°6′25″ E). TY medium (g°L^{-1}: Tryptone, 5; yeast extract, 3; $CaCl_2$, 0.6; agar, 18; pH 7.2) and standard methods described by Vincent (1970) were used for the isolation of root nodule bacteria. Single colonies were picked and repeatedly streaked on TY medium. A total of 82 isolates were screened for copper resistance by growing on TY medium supplied with $CuSO_4$ at final concentrations of 0.2, 0.6, 1.0, 1.4 and 2.0 mM. The bacteria were incubated at 28 °C for both the isolation and screening.

2.2. Preparation of total DNA and plasmid DNA

Strain CCNWSX0020 was incubated in TY broth for 2 days at 28 °C with shaking at 150 rpm. A 3-mL aliquot of the culture was centrifuged at 8000*g* for 5 min with centrifuge Sigma 3 K-15 and the pellet washed by suspension in 1 mL 10 mM Tris–HCl (pH8.0) and centrifugation. Total genomic DNA was extracted following the protocol of Sambrook and Russell (2001). Plasmid DNAs were extracted by the modified alkaline lysis method (Kado and Liu, 1981). Strain CCNWSX0020 was cultured overnight and cells were collected from 2.5 mL culture as described above. The cell pellet was suspended in 50 μL of lysis solution (10% sucrose, 0.01 mg mL^{-1} ribonuclease A, 1 mg mL^{-1} lysozyme) for plasmid separation by electrophoresis (Kado and Liu, 1981). Plasmid pattern from *S. meliloti* 1021 (Galibert et al., 2001) was as reference to estimate the molecular size of plasmids. After staining with ethidium bromide, the plasmids were excised separately under UV light and purified according to their molecular sizes by the method of Sobral et al. (1991).

2.3. PCR amplifications of 16S rRNA, nodA, nifH and copper-resistant genes

The almost complete 16S rRNA gene was amplified from the genomic DNA of strain CCNWSX0020 with primers P1 (5′-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3′) and P6 (5′-TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3′) and the protocol described by Tan et al. (1997). The primers nodA-1 (5′-TGC RGT GGA RDC TRY GCT GGG AAA-3′) and nodA-2 (5′-GGN CCG TCR TCR AAS GTC ARG TA-3′) (Haukka et al., 1998) and nifH-1 (5′-TAC GGN AAR GGS GGN ATC GGC AA-3′) and nifH-2 (5′-AGC ATG TCY TCS AGY TCN TCC A-3′) (Haukka et al., 1998) were used respectively to amplify the *nodA* and *nifH* genes from genomic DNA under the following PCR conditions: an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, elongation at 72 °C for 1 min, with a final extension at 72 °C for 7 min.

For PCR amplification of copper-resistant genes, the DNAs of isolated megaplasmids and chromosome DNA from strain CCNWSX0020 were used separately as templates. The primers amplifying a 1791-bp fragment for *pcoA* (copper-resistant protein A) were pcoA-1 (forward) (5′-CGT CTC GAC GAA CTT TCC TG-3′) and pcoA-2 (reverse) (5′-GGA CTT CAC GAA ACA TTC CC-3′) (Brown et al., 1995). The primers amplifying a 636-bp fragment for *pcoR* (copper-resistant regulatory protein) were pcoR-1 (forward) (5′-CAG GTC GTT ACC TGC AGC AG-3′) and pcoR-2 (reverse) (5′-CTC TGA TCT CCA GGA CAT ATC-3′) (Brown et al., 1995). The conditions for PCR amplification of copper-resistant genes were: an initial denaturation 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C (*pcoA*) and 54 °C (*pcoR*) for 1 min, elongation at 72 °C for 2 min, with a final extension at 72 °C for 7 min.

2.4. DNA sequencing of PCR products and sequence analysis

PCR products were separated by electrophoresis in a 1% (w/v) agarose gel with 0.5× TBE as electrode buffer. PCR bands were excised and purified from the agarose gel using H.Q.&.Q. Gel Extraction Kit (Anhui U-gene Biotechnology Co., Ltd.) and directly ligated into the pGEM®-T Easy vector (Promega). *E. coli* DH5α competent cells were transformed and plated onto LB agar with ampicillin (100 μg mL^{-1}), X-Gal (40 μg mL^{-1}) and IPTG (24 μg mL^{-1}). DNA sequences of both strands were determined using T7 and SP6 primers and an ABI model 377 Stretch automated DNA sequencer (Perkin-Elmer Applied Biosystems). The nucleotide sequence and deduced protein sequences were compared with sequences in GenBank using BLAST (blastn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the strain was identified by the sequence comparison.

2.5. Production of IAA, siderophore and ACC deaminase

To determine IAA (indole acetic acid) production, strain CCNWSX0020 was incubated in 10 mL of SMS medium (g L^{-1}: sucrose, 10.0; $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.5; yeast extract, 0.5; $CaCO_3$, 0.5; NaCl, 0.1; pH 7.2) supplemented with 0.5 mg mL^{-1} of typtophan for 5 days at 28 °C with shaking at 150 rpm. Cells and supernatants were separated by centrifugation at 10 000*g* for 5 min, and 1 mL of supernatant was mixed with 100 μL 10 mM orthophosphoric acid and 2 mL of Salkowski reagent (Gordon and Weber, 1951). After incubation for 30 min at 25 °C, the absorbance was determined at 530 nm. The IAA concentration in culture was determined from the calibration curve using standard IAA solution.

Siderophore production was detected using Chrome azurol S (CAS) plates (Schwyn and Neilands, 1987) and ACC deaminase activity was measured according to the protocol of Tittabutr et al. (2008) by spectrometric measuring the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC in YM broth (Vincent, 1970).

2.6. Cu^{2+} biosorption and bioaccumulation assays

2.6.1. Preparation of the bacterial biosorbent

Strain CCNWSX0020 was pre-cultured in TY medium at 28 °C for 48 h with shaking at 150 rpm. The cells were harvested by centrifugation at 10 000*g* for 8 min from early-stationary cultures (OD_{600} = 1.2–1.5), and the bacterial biosorbent was adjusted to a cell density of approximately 2.0 g dry weight per liter. The cells were rinsed three times with sterilized ddH_2O, and then re-suspended in designated heavy-metal solutions for biosorption experiments.

2.6.2. Time-course of biosorption

The rhizobial biosorbent was suspended in 50 mL of 100 mg L^{-1} of Cu^{2+} solutions in an Erlenmeyer flask with cell concentration of 2.0 g L^{-1}. To avoid precipitation of metals in the form of metal hydroxides, the pH of the Cu^{2+} solutions was initially adjusted to 5.0 with 0.1 M HCl (Chang et al., 1997). The cell-metal suspension was agitated at 100 rpm at 28 °C. Samples were taken from the solutions at intervals and centrifuged at 10 000*g* for 8 min. The copper ions in the supernatant were measured by atomic absorption spectrometry (AAS) and the biosorption capacity q_e (mg metal g^{-1} dry cell) was calculated as $q_e = (C_0 - C_e)/X$, where C_0 is the initial metal concentration (mg L^{-1}), C_e is the residual Cu^{2+} concentration (mg L^{-1}) and X is the biomass concentration (g dry cell L^{-1}).

2.6.3. Determination of biosorption isotherms

At a dosage of 2.0 g L^{-1}, the biosorbent was suspended in Cu^{2+} solutions with an initial concentration ranging from 0 to 100 mg L^{-1}. The residual Cu^{2+} concentration in the suspensions was measured with AAS after 24 h incubation at 28 °C and 100 rpm agitation. The experimental results were simulated by