Genes Conferring Copper Resistance in *Sinorhizobium meliloti* CCNWSX0020 Also Promote the Growth of *Medicago lupulina* in Copper-Contaminated Soil

Zhefei Li,^a Zhanqiang Ma,^a Xiuli Hao,^a Christopher Rensing,^b Gehong Wei^a

State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, China^a; Department of Plant and Environmental Science, University of Copenhagen, Frederiksberg, Denmark^b

Sinorhizobium meliloti CCNWSX0020, isolated from root nodules of *Medicago lupulina* growing in gold mine tailings in the northwest of China, displayed both copper resistance and growth promotion of leguminous plants in copper-contaminated soil. Nevertheless, the genetic and biochemical mechanisms responsible for copper resistance in *S. meliloti* CCNWSX0020 remained uncharacterized. To investigate genes involved in copper resistance, an *S. meliloti* CCNWSX0020 Tn5 insertion library of 14,000 mutants was created. Five copper-sensitive mutants, named SXa-1, SXa-2, SXC-1, SXC-2, and SXn, were isolated, and the disrupted regions involved were identified by inverse PCR and subsequent sequencing. Both SXa-1 and SXa-2 carried a transposon insertion in *lpxXL* (SM0020_18047), encoding the LpxXL C-28 acyltransferase; SXC-1 and SXC-2 carried a transposon insertion in *merR* (SM0020_29390), encoding the regulatory activator; SXn contained a transposon insertion in *omp* (SM0020_18792), encoding a hypothetical outer membrane protein. The results of reverse transcriptase PCR (RT-PCR) combined with transposon gene disruptions revealed that SM0020_05862, encoding an unusual P-type ATPase, was regulated by the MerR protein. Analysis of the genome sequence showed that this P-type ATPase did not contain an N-terminal metal-binding domain or a CPC motif but rather TPCP compared with CopA from *Escherichia coli*. Pot experiments were carried out to determine whether growth and copper accumulation of the host plant *M. lupulina* were affected in the presence of the wild type or the different mutants. Soil samples were subjected to three levels of copper contamination, namely, the uncontaminated control and 47.36 and 142.08 mg/kg, and three replicates were conducted for each treatment. The results showed that the wild-type *S. meliloti* CCNWSX0020 enabled the host plant to grow better and accumulate copper ions. The plant dry weight and copper content of *M. lupulina* inoculated with the 5 copper-sensitive mutants significantly decreased in the presence of CuSO₄.

Copper is an essential trace element for most living organisms, since it is a constituent of many metalloenzymes and proteins involved in electron transport, redox, and other important reactions (1). Copper is usually cytotoxic at high concentration, it persists in the environment, and it is risky to living organisms (2, 3). Copper may reduce biodiversity and significantly limit reproduction, growth, and activity of bacteria, i.e., nitrogen fixation in diazotrophs (4). Transport systems for regulating copper homeostasis play a crucial role in tolerating copper toxicity in most organisms. These copper homeostasis mechanisms involve uptake, efflux, and sequestration of copper (5–8). In addition, a global adaptive response involving induction of other stress regulons is also frequently presented (9–11). However, the mechanisms used and particularly the coordination of regulatory responses vary significantly between species.

Metal tailings are highly polluted areas with extremely high levels of heavy and transition metal content in the soil, and those areas are also deficient in water and nutrients (12). Such harsh conditions make copper-contaminated soil highly disadvantageous for the survival of various plants which could restore soil function through the accumulation of metals in its tissues. Legumes, and their associated rhizobial bacteria, are important components of the biogeochemical cycles in agricultural and natural ecosystems. Rhizobia are Gram-negative soil bacteria that have economic value and agronomic significance due to their ability to establish a nitrogen-fixing symbiosis with leguminous plants. However, many environmental factors, including heavy and transition metals, often limit the potential of symbiotic sys-

tems and have negative effects on both growth and nitrogen fixation of rhizobia (13). Recently, many rhizobial strains with enhanced ability to survive in the presence of high concentrations of heavy and transition metals have been isolated from the root nodules of various legumes (14, 15). Further studies revealed that the nodulation and nitrogen-fixing ability of metal-resistant rhizobia were not affected by metal ions. Moreover, some of them secreted siderophores, 1-aminocyclopropane-1-carboxylate deaminase (ACC), and indole-3-acetic acid (IAA) (16, 17). Therefore, rhizobia play a key role in host plant adaptation to heavy and transition metal-contaminated soils through physiological changes of the plant metabolism and could have enormous potential in the use of plant-microorganism interactions to aid bioremediation of contaminated soils.

Sinorhizobium meliloti CCNWSX0020, isolated from root nodules of *Medicago lupulina* growing in gold mine tailings in the northwest of China, was resistant to 1.4 mM Cu²⁺ in tryptone-yeast extract (TY) medium (1.8 mM Cu²⁺ in yeast-mannitol agar

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Address correspondence to Gehong Wei, weigehong@nwsuaf.edu.cn.

Z.L. and Z.M. contributed equally to this work.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
<i>S. meliloti</i> CCNWSX0020	Wild-type strain, Nod ⁺ on <i>Medicago lupulina</i> , Amp ^r	This work
SXa-1 (<i>lpxXL::Tn5</i>)	Copper-sensitive mutant carrying Tn5-1063a	This work
SXa-2 (<i>lpxXL::Tn5</i>)	Copper-sensitive mutant carrying Tn5-1063a	This work
SXc-1 (<i>merR::Tn5</i>)	Copper-sensitive mutant carrying Tn5-1063a	This work
SXc-2 (<i>merR::Tn5</i>)	Copper-sensitive mutant carrying Tn5-1063a	This work
SXn (<i>omp::Tn5</i>)	Copper-sensitive mutant carrying Tn5-1063a	This work
<i>E. coli</i> DH5α	<i>lacZΔM15 recA1 gyrA96 hsdR17</i>	51
<i>E. coli</i> S17-1	<i>recA pro tra⁺</i>	52
Plasmids		
pRL1063a	Carrying Tn5- <i>luxAB</i> , Km ^r Sm ^r	Michigan State University, Peter Wolk
pRK2013	Helper plasmid, Km ^r	University of York, United Kingdom, Tanya Soule
pGEM-T easy	Cloning and sequencing vector, Amp ^r	Promega

[YMA] medium). The genome of *S. meliloti* CCNWSX0020 was determined in our work (18). Using scanning electron microscopy and infrared spectrum, in our previous work we found that the bacterium accumulated Cu²⁺ mainly on the cell surface (19). So far, there is scarce knowledge about genetically determined mechanisms of copper resistance in *S. meliloti* CCNWSX0020 which colonizes plants in copper-contaminated soil. Therefore, the aim of this study was to identify genes involved in copper resistance/homeostasis of this strain through transposon mutagenesis combined with transcription analysis via reverse transcriptase PCR.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids in this work are shown in Table 1. *Sinorhizobium meliloti* CCNWSX0020 was grown at 28°C in tryptone-yeast extract medium (TY) or yeast-mannitol agar (YMA) (20). *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) medium. Antibiotics were used at the following concentrations: 100 µg/ml ampicillin (Amp) for *S. meliloti* CCNWSX0020; 50 µg/ml kanamycin (Km) for *E. coli*; a combination of 100 µg/ml Amp and 50 µg/ml Km for the mutants; 20 µg/ml gentamicin (Gm) for complementation studies.

Random transposon mutagenesis and screening for copper-sensitive mutants. Transposon insertion mutagenesis was conducted using *S. meliloti* CCNWSX0020 (Amp^r) as the recipient, *E. coli* DH5α cells containing the suicide plasmid pRL1063a (Km^r) as the donor, and *E. coli* DH5α cells containing pRK2013 as a helper in triparental mating. A random insertion mutant library was generated. The mutagenized cells were plated on TY medium containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml). Colonies resistant to both antibiotics were picked up by toothpicks and then streaked onto TY plates with a final concentration of 0.8 mM Cu²⁺. Clones that were unable to grow or grew poorly in the presence of 0.8 mM Cu²⁺ but grew well on TY plates were subjected to further analysis.

Growth in metal-containing medium. *Sinorhizobium meliloti* CCNWSX0020 and copper-sensitive mutants were precultured in TY medium at 28°C with shaking at 150 rpm for 48 h, and 1% of the cultures was added to TY medium (pH = 7.0) supplemented with four different metals at the following concentrations: CuSO₄, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mM; ZnSO₄, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mM; CdCl₂, 0.05, 0.1, 0.2, 0.3, 0.4; Pb(NO₃)₂, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mM. The cells were then incubated again for 48 h with shaking at 150 rpm, and growth was determined by measuring the optical density at 600 nm.

DNA manipulations and sequence analysis. Copper-sensitive mutants were incubated for 2 days at 28°C in TY broth with shaking at 150 rpm. A 3-ml aliquot of the culture was centrifuged at 8,000 × g for 5 min

with a Sigma 3K-15 centrifuge, and the pellet was washed by suspension in 1 ml 10 mM Tris-HCl (pH 8.0) and subsequent centrifugation. Genomic DNA was extracted by following the protocol of Wilson and Carson (21). Digestions by restriction enzymes, ligations, and transformations were performed using standard methods (22). To determine a single copy of the transposon in mutants, Southern blotting experiments were performed. Fragments of digested DNA were separated on 0.7% agarose gels and blotted onto charged nylon membranes. DNA hybridizations were performed by using a biotinylated DNA labeling of pRL1063a and a detection kit according to the manufacturer’s instructions (Roche Diagnostics). To recover *S. meliloti* genomic sequences contiguous with the inserted transposon, genomic DNA from each *S. meliloti* CCNWSX0020::Tn5 pRL1063a mutant was digested by EcoRI or ClaI, which did not cut within the transposon. Digested DNA was self-ligated using the 100 lig Fast-Link DNA ligation kit (Epicentre, Madison, WI) at room temperature for 20 min and transformed into *E. coli* DH5α-competent cells where *oriV* in pRL1063a maintained the DNA as a plasmid. Cells were recovered for 1 h in LB broth at 37°C with shaking at 150 rpm and plated onto LB plates with kanamycin (50 µg ml⁻¹) to select for cells transformed with the ligated pRL1063a plasmid containing flanking *S. meliloti* DNA. The DNA sequence of the *S. meliloti* fragments was determined by using outward primers corresponding to the left and right ends of Tn5 pRL1063a as described previously (23). The nucleotide sequence and deduced protein sequences (Omp, EHK76610.1; LpxXL, EHK78957.1; MerR, EHK74354.1) were compared with sequences in GenBank using BLAST.

Complementation test of mutants. To complement the mutant phenotypes, the genes which contain the regulatory region were amplified from genomic DNA of *S. meliloti* CCNWSX0020. PCR amplification of *omp*, *lpxXL*, and *merR* was performed using primers (*omp*, 5'-TTGGTA CCATCCCGAGGAACGGAGAA and 5'-CCATCGATGCTCACGAAG AAGCCCATCA; *merR*, 5'-TTCTCGAGGGCCGAGATAGGCTGAACG and 5'-CG GAATTCAAGATGCGGAGCGAAAGT; *lpxXL*, 5'-TTGGTA CCGCGAAGAAGAA GGGCGTAG and 5'-GCATCGATAACGGCACC GATTAAAGGA), and the production was cloned into the KpnI/ClaI or XhoI/EcoRI site of the broad-range plasmid pBBR1-MCS5. The underlined regions represent the restriction sites. Complementation constructs were sequence verified with the corresponding primers, transformed into *E. coli* donor strain JM109, and delivered into mutants via triparental mating. Single colonies carrying the complementation plasmids were selected on YMA plates with gentamicin and assayed for growth in medium supplemented with different concentrations of CuSO₄.

Validation of candidate Cu-resistant genes by RT-PCR. *Sinorhizobium meliloti* CCNWSX0020 and its copper-sensitive mutants SXc-1 and SXn were cultured in TY medium separately. Culture solutions were supplemented with Cu²⁺, Pb²⁺, Zn²⁺, Cd²⁺, and Mn²⁺ at a concentration of

0.5 mM and Ag⁺ at a concentration of 0.2 mM for 15 min, respectively, when bacterial density reached an optical density at 600 nm (OD₆₀₀) of 0.5. Total RNA was extracted, and at the same time, trace genomic DNA was degraded by DNase I (TaKaRa). Reverse transcription and reverse transcriptase PCR (RT-PCR) were performed using the TaKaRa reverse transcription kit and SYBR Premix Ex Taq II (Tli RNaseH Plus) kit. Quantitative RT-PCR was performed using distilled water instead of first-strand cDNA as a template in the negative control experiment. Amplification was performed on a Bio-Rad CFX96 thermal cycler at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. To correct for differences in the amount of starting material, 16S rRNA was used as the endogenous reference gene. Results were presented as ratios of gene expression between the target gene and the reference gene, and an increase of 4-fold or more compared to *S. meliloti* CCNWSX0020 was considered overexpression.

Genome sequence analysis. The draft genome of *S. meliloti* CCNWSX0020 was sequenced and annotated (accession number AGVV000000000), and genes and protein sequences were submitted to NCBI in a previous work (18). Copper-resistant and plant growth-promoting gene function were identified using BLAST in the RAST server. Metabolic pathways were predicted according to a KEGG pathway map.

Plant growth, nodulation conditions, and IAA determination. *Medicago lupulina* seeds were sterilized in the following conditions: 95% ethanol for 1 min, 5% sodium hypochlorite for 3 min, and sterile distilled water for surface sterilization. Sterilized seeds were germinated in petri dishes with water agar at 28°C for 48 h, and then seedlings were sown in pots filled with 135 ± 1 g sterilized perlite-vermiculite (3:2) supplied with 0, 47.36, and 142.08 mg/kg of CuSO₄ and were incubated in the greenhouse at 25°C. The log-phase cells of *S. meliloti* CCNWSX0020 or mutants were washed three times with sterile water, and the cells were resuspended in sterile water (approximately 1 × 10⁸ CFU/ml). The bacterial suspension was inoculated into pots out of which the first true leaves of *Medicago lupulina* seedlings grew. Seedlings without inoculation were included as blank controls. Plants were harvested after 5 weeks, and the quantity of pink and effective nodules on plant roots inoculated with *S. meliloti* and the dry weight of shoots and roots were determined. Afterward, shoots and roots were dried at 80°C and grounded. The samples were digested with nitric acid-perchloric acid mixture (HNO₃ and HClO₄ in a 5:1 ratio), and copper content was analyzed by atomic absorption spectrophotometry. IAA production of *S. meliloti* CCNWSX0020 and mutants was monitored for 2 days at 28°C with shaking at 180 rpm as described previously (24), and IAA concentrations were determined by the Salkowski reaction (25).

Statistical analysis. Statistical analyses were performed with SAS v8 software. Data were compared by analysis of variance and multiple comparison tests (Duncan's shortest significant ranges [SSR]).

RESULTS

Copper-sensitive mutants could be obtained by transposon mutagenesis. *Sinorhizobium meliloti* CCNWSX0020, isolated from root nodules of *M. lupulina* growing in gold mine tailings in the northwest of China, was resistant up to 1.4 mM CuSO₄ in TY medium. To isolate copper-sensitive mutants, Tn5-1063a was used to mutate *S. meliloti* CCNWSX0020. A total of 14,000 Km^r transconjugants were analyzed, of which five mutants, named SXa-1, SXa-2, SXC-1, SXC-2, and SXn, were unable to grow on TY agar plates supplemented with 0.8 mM CuSO₄. Probing the total genomic DNA of these mutants (digested with BglII) with a labeled Tn5 DNA fragment established that each strain carried a single Tn5-*luxAB* insertion (data not shown) (23).

Metals specifically affected growth of the mutants. Growth of the wild type and its mutants were analyzed in TY medium with different CuSO₄ concentrations. Mutants SXC-1, SXC-2, and SXn were unable to grow in the presence of 0.6 mM CuSO₄ (Fig. 1A).

In contrast, strains SXa-1 and SXa-2 showed sensitivity to Cu²⁺ only at the higher concentrations of CuSO₄ (0.8 to 1.0 mM) (Fig. 1A). In addition, all mutants tested in the presence of Zn²⁺, Cd²⁺, and Pb²⁺ were shown to be sensitive to these metals. However, the patterns of sensitivity among the five mutants varied. SXC-1, SXC-2, and SXn were the most sensitive to Zn²⁺, and SXa-1 and SXa-2 showed minimal sensitivity to Zn²⁺. Strains SXC-1, SXC-2, and SXn were more sensitive to Pb²⁺ than the wild-type strain. However, all five mutants showed a high degree of sensitivity to Cd²⁺ (Fig. 1B, C, and D).

Genes responsible for copper homeostasis could be identified. Genomic DNA was isolated from the mutants, digested with EcoRI or ClaI, self-ligated, and transformed into *E. coli* DH5a (26). The five copper-sensitive insertions and the flanking gene sequences were sequenced and shown to belong to three different genes (Table 2). The genes and the relative location of the transposon insertions were determined (Fig. 2). In strains SXa-1 and SXa-2, the interrupted gene was 99.1% identical to *lpxXL*, encoding LpxXL C-28 acyltransferase previously identified in *S. meliloti* 1021 (27). In strains SXC-1 and SXC-2, the interrupted gene had the highest identity with the *merR* gene of *S. meliloti* 1021. In strain SXn, the Tn5 insertion interrupted *omp* encoding a protein highly similar to a hypothetical protein, which showed 98% similarity to a putative outer membrane efflux protein in *Rhizobium leguminosarum* bv. *viciae* 3841 (28). To verify whether these genes were able to recover the copper resistance of these mutants or not, the genes, including the promoter of *lpxXL*, *merR*, and *omp*, were amplified and inserted into the pBBR1 MCS-5 vector and then transformed into the corresponding mutant (designated pMC-SXa-1-1, pMC-SXC-1-1, and pMC-SXn-1) and tested for copper tolerance. Copper resistance of *omp*, *merR*, and *lpxXL* mutants could be restored to a certain extent (Table 3). These results suggested that copper sensitivity of the mutants was due to the insertional disruption of these particular genes in *S. meliloti* CCNWSX0020.

Symbiotic properties of mutants. Each of the mutants was used to inoculate alfalfa plants, and all the mutants could nodulate with *M. lupulina* (Table 4). The plants inoculated with *S. meliloti* CCNWSX0020 had an average of 11 nodules per root, and there was no decline in nodule numbers of alfalfa plants inoculated with each mutant. However, the plants inoculated with SXa-1 and SXa-2 had an average of 11 and 10 nodules per plant, respectively, and about 45% and 40% of the nodules were white and ineffective. These data suggested that the normal development of the nitrogen-fixing nodules was affected. In contrast, the nodules of plants inoculated with SXC-1, SXC-2, and SXn mutants were pink and cylindrical after 6 weeks. Therefore, the SXC-1, SXC-2, and SXn mutants had the capacity to establish the symbiotic association with *M. lupulina* successfully.

Expression of genes conferring Cu resistance. The SXC-1 and SXC-2 (*merR*::Tn5) mutants were not only sensitive to Cu²⁺ but also to Zn²⁺, Cd²⁺, and Pb²⁺. Since previous reports demonstrated that glutathione was involved in resistance to a number of transition and heavy metals (29), RT-PCR was performed to detect expression of *SM0020_29380* and *SM0020_29385*, encoding SAM-dependent methyltransferase and glutaredoxin, adjacent to *merR*. However, we failed to notice the expression of these two genes changing with induction by Zn²⁺, Cd²⁺, or Pb²⁺ in both the wild-type and the SXC mutants. In addition, a conserved MerR binding region was not found in the upstream of *SM0020_29380* and *SM0020_29385*.

FIG 1 Growth of *S. meliloti* CCNWSX0020 and the mutants which had been incubated for 48 h with the different heavy metals. (A) Copper; (B) zinc; (C) cadmium; (D) lead. Also shown are *S. meliloti* CCNWSX0020 (■), SXa-1 (◆), SXa-2 (▲), SXc-1 (●), SXc-2 (△), and SXn (◇).

Surprisingly, the expression of *SM0020_29380* and *SM0020_29385* was highly induced by Cu^{2+} in both wild-type and SXc mutants (Fig. 3A and B). The observations implied that the two genes were involved only in copper resistance, but MerR in *S. meliloti* CCNWSX0020 was found not to regulate the expression of these two genes. The expression of *SM0020_05727* and *SM0020_05862*, encoding putative cation transport P-type ATPases, could be induced by Cu^{2+} , Zn^{2+} , Cd^{2+} , and Pb^{2+} in the wild type; however, the expression of these two genes declined sharply in the SXc mutants (Fig. 4).

In the mutant SXn (*omp::Tn5*), *omp* (*SM0020_18792*) is part of an operon where *SM0020_18782* and *SM0020_18787* both encode unknown proteins; *SM0020_18797* encodes a multicopper oxidase, *SM0020_18802* encodes a blue copper azurin-like protein, and *SM0020_18807* encodes a potential periplasmic copper chaperone. When *S. meliloti* CCNWSX0020 and SXn were cultured in TY medium supplemented with Cu^{2+} at the concentration of 0.5

mM, the expression of the six genes was increased and was further increased in the mutant compared to the wild-type, and the expression of *SM0020_18787* and *SM0020_18797* was slightly higher than that of the other genes (Fig. 5).

Putative copper resistance genes identified in the draft genome of *S. meliloti* CCNWSX0020. The draft genome sequence of *S. meliloti* CCNWSX0020 comprises 7,001,588 bases representing a 22-fold coverage of the genome. The genome of *S. meliloti* CCNWSX0020 has a G + C content of 59.9%. There are a total of 7,086 genes, including 6 rRNA genes, 47 tRNA genes, and 7,033 putative protein-coding sequences (CDSs), within the genome of *S. meliloti* CCNWSX0020. Among the predicted CDSs, 6,206 are similar to genes found in *S. meliloti* 1021. Additionally, *S. meliloti* CCNWSX0020 carried a number of predicted protein-coding genes involved in copper homeostasis: two operons encoding putative copper tolerance proteins, two encoding putative multicopper oxidases, one encoding a blue copper oxidase precursor, six

TABLE 2 Identification of Tn5-1063a insertion sites and identity to open reading frame sequences from the NCBI databases

Mutant strain	Open reading frame location in <i>S. meliloti</i> CCNWSX0020	Gene (length in bp)	Insertion site (bp)	Predicted gene product	Closest identity (%)
SXa-1	<i>SM0020_18047</i>	<i>lpxXL</i> (939)	64–65	LpxXL C-28 acyltransferase	99.1
SXa-2	<i>SM0020_18047</i>		331–332		
SXc-1	<i>SM0020_29390</i>	MerR-like gene (405)	88–89	Transcriptional regulator, MerR/CueR family	99.6
SXc-2	<i>SM0020_29390</i>		260–261	Transcriptional regulator, MerR/CueR family	
SXn	<i>SM0020_18792</i>	Unknown (1,446)	541–542	Conserved hypothetical protein	98.8

FIG 2 Relative transposon insertion locations in putative genes of the SXa-1 and SXa-2 mutants (a), SXC-1 and SXC-2 mutants (b), and SXn mutant (c). Genes flanking these loci and their orientations are also shown.

encoding putative transition or heavy metal-translocating P-type ATPases with a typical CPx metal binding motif in the sixth transmembrane helix, one encoding the copper homeostasis protein CutE, and one encoding the cytoplasmic copper homeostasis protein CutC (data not shown). These features may contribute to the heavy and transition metal resistance in *S. meliloti* CCNWSX0020.

Plant growth and Cu^{2+} content in the tissue of *M. lupulina* were influenced by mutants influencing copper resistance in *S. meliloti* CCNWSX0020. Plant growth and Cu^{2+} accumulation in the shoots and roots of *M. lupulina* were significantly influenced by the inoculation of *S. meliloti* CCNWSX0020 or the copper-sensitive mutants (Tables 5 and 6). The growth of *M. lupulina* without inoculation of *S. meliloti* CCNWSX0020 was significantly inhibited at a high concentration of CuSO_4 , as the color of the plant turned to light brown and part of the leaves changed due to chlorosis on the fourteenth day (data not shown). In addition, high concentrations of CuSO_4 ($142.08 \text{ mg kg}^{-1}$) inhibited the growth of *M. lupulina* inoculated with copper-sensitive mutants, but the plants were healthy when inoculated with *S. meliloti*

CCNWSX0020. Significant decreases ($P < 0.05$) in root and shoot dry weight of *M. lupulina* inoculated with copper-sensitive mutants were observed compared with those inoculated with *S. meliloti* CCNWSX0020. The root and shoot dry weights of *M. lupulina* inoculated with the SXa-1, SXC-1, and SXn mutants were 50%, 56%, and 26% and 67.44%, 72.09%, and 26.74% less than those inoculated with *S. meliloti* CCNWSX0020 in the presence of $142.08 \text{ mg/kg CuSO}_4$, respectively. The reduction of *M. lupulina* root and shoot dry weight also displayed the same trend when inoculated with the SXa-2 and SXC-2 mutants. In addition, the biomass of *M. lupulina* inoculated with SXa-1, SXC-1, and SXn mutants in the presence of $142.08 \text{ mg kg}^{-1} \text{ CuSO}_4$ decreased by 61.03%, 66.18%, and 26.47%, respectively. As the Cu^{2+} concentrations in soil increased, the Cu^{2+} content in roots and shoots followed a consistent trend. However, inoculation with copper-sensitive mutants significantly decreased the Cu^{2+} content in roots and shoots of *M. lupulina*, compared with the plants inoculated with the *S. meliloti* CCNWSX0020 strain ($P < 0.05$).

TABLE 3 Complementation of mutants at different concentrations of CuSO_4

Strain	Complementation ^a at concn (mmol/liter) of:						
	0	0.3	0.6	0.9	1.2	1.5	1.8
<i>S. meliloti</i> CCNWSX0020	+	+	+	+	+	+	+
pMC-SXa-1-1	+	+	+	+	+	+	—
pMC-SXC-1-1	+	+	+	+	+	+	—
pMC-SXn-1	+	+	+	+	+	+	—

^a All bacteria grown in YMA medium complement with different concentrations of CuSO_4 . +, bacteria grew; —, bacteria did not grow.

TABLE 4 Effect of *S. meliloti* CCNWSX0020 mutants on *M. lupulina*^a

Bacterial strain	Symbiosis	No. of pink nodules	No. of white nodules	Production of IAA ($\mu\text{g/ml}$)
<i>S. meliloti</i> CCNWSX0020	Yes	11 $\pm$ 4	0	13.2 $\pm$ 1.1
SXa-1	Yes	6 $\pm$ 2	5 $\pm$ 2	9.1 $\pm$ 0.9
SXa-2	Yes	6 $\pm$ 3	4 $\pm$ 5	9.4 $\pm$ 0.8
SXC-1	Yes	11 $\pm$ 5	0	7.3 $\pm$ 1.2
SXC-2	Yes	10 $\pm$ 3	0	8.2 $\pm$ 0.8
SXn	Yes	10 $\pm$ 2	0	5.7 $\pm$ 0.9

^a Values are the means ($n = 6$ experiments) $\pm$ 1 standard error.

FIG 3 (A and B) Quantification of *SM0020_29380* and *SM0020_29385* fragments. 0020 was the wild type; SXC-1 was the *merR* mutant. The expression of these genes was induced by 0.5 mM copper, zinc, lead, manganese, and cadmium. *SM0020_29380* code SAM-dependent methyltransferase and *SM0020_29385* codes glutaredoxin and related proteins.

DISCUSSION

A number of transition metals are required by plants as essential elements which are involved in a wide variety of metabolic pathways. However, transition metals are toxic to plants if the concentration exceeds normal levels (2). Therefore, utilizing hyperaccumulators to restore the transition metal-contaminated soil has received special attention (30, 31). Unfortunately, transition metals can be toxic for metal-hyperaccumulating plants if the concentration of metals in the environment is sufficiently high. In addition, heavy and transition metal-contaminated soil is often deficient in nitrogen nutrition, water, and iron (32, 33). *S. meliloti* CCNWSX0020, isolated from root nodules of *M. lupulina* growing in gold mine tailings in the northwest of China, displayed transition metal resistance. Results of construction and screening of a transposon insertion library showed that interruption of *merR* (*SM0020_29390*) encoding an MerR family transcriptional regulator, *lpxXL* (*SM0020_18047*) encoding an LpxXL C-28 acyltransferase, and *omp* (*SM0020_18792*) encoding a hypothetical outer membrane protein decreased Cu^{2+} resistance of *S. meliloti* CCNWSX0020 dramatically. There are two transcriptional regulators (*SM0020_11410* and *SM0020_29390*) of the MerR family in strain *S. meliloti* CCNWSX0020. The gene downstream of *SM0020_*

11410 encodes a P-type ATPase which is highly similar to the ActP protein from *Sinorhizobium fredii* NGR234 (66% identity over 802 amino acids). The rhizobial ActP protein would therefore be exporting excess copper from the cell in response to toxic copper concentrations (34). Combined with the results of quantitative RT-PCR analysis, two genes (*SM0020_05727* and *SM0020_05862*) encoding putative cation transport P-type ATPases were found to downregulate in the *merR* (*SM0020_29390*)-interrupted mutant. The SXC-1 and SXC-2 mutants were more sensitive than the other mutants, suggesting that putative cation transport P-type ATPases were the primary system for Cu^{2+} resistance in *S. meliloti* CCNWSX0020. An interesting observation was that the SXC-1 and SXC-2 were also sensitive to Cu^{2+} , Pb^{2+} , Cd^{2+} , and Zn^{2+} ions. Usually copper efflux $\text{P}_{1\text{B}}$ -type ATPase contains an N-terminal CXXC motif (LSGMSASCVRVQNALQSVPGVTQARVNL), histidine-rich leader sequences, or methionine-rich leader sequences (35, 36). However, this metal-binding motif is not present in these putative cation transport $\text{P}_{1\text{B}}$ -type ATPases encoded by *SM0020_05727* and *SM0020_05862*. In addition, the gene product of *SM0020_05862* is a highly unusual $\text{P}_{1\text{B}}$ -type ATPases since it does not contain a typical CPX motif but rather TPCP(X)₅P (Fig. 6). Arguello (37) suggested that protein in this motif and Gln, Glu, Ser, and Asp residues in transmembranes 7 and 8 may be relevant to metal specificity, but this still has to await further determination. So this $\text{P}_{1\text{B}}$ -type ATase might transport a number of metals comparatively unspecific as judging from the phenotype.

The *lpxXL* (*SM0020_18047*) encoded a C-28 acyltransferase which transfers very-long-chain fatty acid (VLCFA) from VLCFA-AcpXL to Kdo2-lipid IV_A, which forms the penta-acylated lipid A molecule. C-28 acyltransferase plays an important but distinct role in *S. meliloti* bacteroid development during symbiosis with alfalfa. The *lpxXL* mutant lacking lipid A VLCFAs has reduced competitiveness in alfalfa (38). Research has shown that penta-acylated lipid molecules modified with VLCFA or unhydroxylated fatty acid can interact with divalent cations and improve cell membrane stability. It is therefore important for an increased cell resistance to a wide range of environmental stress, such as salt, detergents, and osmotic resistance (39, 40). Our study found that VLCFA-modified penta-acylated lipid molecules were able to enhance resistance to metals such as copper, zinc, and cadmium. Divalent cations can stabilize the outer membrane by interacting with lipopolysaccharide (LPS) (41). This suggested that the absence of penta-acylated lipid A in the *lpxXL* mutant could weaken the outer membrane stability, thereby resulting in poor growth on TY agar supplemented with Cu^{2+} and other metals.

Based on the genome sequence of *S. meliloti* CCNWSX0020, various genes might be involved in copper homeostasis and resistance. The genes *cueO* (*SM0020_18797*, *SM0020_23122*) and *copA* (*SM0020_11415*, *SM0020_05912*, *SM0020_05727*), encoding a periplasmic multicopper oxidase and a copper transport P-type ATPase, respectively, resemble the CopA/Cue system. Under aerobic conditions, the copper-sensing transcriptional regulator activates the expression of *copA* and *cueO*. While CopA pumps out excessive copper from the cytoplasm into the periplasm, CueO oxidizes Cu^+ to Cu^{2+} in the periplasm, thereby reducing copper toxicity in *E. coli*. But whether the cells detoxicate toxicity of copper in this way needs to be confirmed in *S. meliloti*. In addition, copper chaperones (encoded by *SM0020_05305*, *SM0020_11420*), copper-binding proteins (encoded by *SM0020_14779*, *SM0020_*

FIG 4 Quantification of *SM0020_05727* and *SM0020_05862* fragments. 0020 was the wild type; SXc-1 was the *merR* mutant. The expression of these genes was induced by 0.5 mM copper, zinc, lead, and cadmium. These two genes code cation transport P-type ATPase.

30972, and *SM0020_18802*), and cation transport P-type ATPases (encoded by *SM0020_05912*, *SM0020_05862*, and *SM0020_05727*) may participate in copper transport and sequestration. Furthermore, *SM0020_05727* and *SM0020_05862* lack the HMA

domain at the N terminus. Therefore, the specific functions of the various cation transport P-type ATPases in *S. meliloti* CCNWSX0020 need to be determined experimentally.

The SOD and CAT activity of cells also increased with elevated

FIG 5 Quantification of gene fragments around *omp*. 0020 was the wild type; SXn was the *omp* mutant. The expression of these genes was induced by the addition of copper (at a final concentration of 0.5 mM) or silver (at a final concentration of 0.2 mM) for 15 min. *SM0020_18782* and *SM0020_18787* encode an unknown protein, *SM0020_18792* encodes an outer membrane protein, *SM0020_18797* encodes a multicopper oxidase, *SM0020_18802* encodes a blue copper azurin-like protein, and *SM0020_18807* encodes a periplasmic copper chaperone.

TABLE 5 Effect of mutants on the root and shoot dry weights of *M. lupulina* on perlite-vermiculite added with different concentrations of CuSO₄

Strain	Dry wt of roots or shoots of <i>M. lupulina</i> with different treatments ^a					
	Root dry wt			Shoot dry wt		
	0 mg/kg CuSO ₄	47.36 mg/kg CuSO ₄	142.08 mg/kg CuSO ₄	0 mg/kg CuSO ₄	47.36 mg/kg CuSO ₄	142.08 mg/kg CuSO ₄
Control	0.47 ± 0.06 C	0.33 ± 0.02 C	0.21 ± 0.02 C	0.70 ± 0.03 B	0.49 ± 0.02 C	0.23 ± 0.01 C
<i>S. meliloti</i> CCNWSX0020	0.65 ± 0.03 A	0.70 ± 0.02 A	0.50 ± 0.02 A	0.97 ± 0.02 A	1.12 ± 0.01 A	0.86 ± 0.02 A
SXa-1	0.54 ± 0.02 BC	0.39 ± 0.02 B	0.25 ± 0.01 C	0.79 ± 0.02 B	0.53 ± 0.01 C	0.28 ± 0.01 C
SXa-2	0.51 ± 0.02 BC	0.41 ± 0.01 B	0.26 ± 0.02 C	0.76 ± 0.02 B	0.51 ± 0.02 C	0.27 ± 0.01 C
SXc-1	0.59 ± 0.03 AB	0.35 ± 0.02 B	0.22 ± 0.01 C	0.90 ± 0.01 AB	0.49 ± 0.02 C	0.24 ± 0.02 CD
SXc-2	0.61 ± 0.03 AB	0.33 ± 0.01 B	0.20 ± 0.02 C	0.92 ± 0.02 AB	0.50 ± 0.01 C	0.26 ± 0.02 C
SXn	0.61 ± 0.02 AB	0.65 ± 0.02 A	0.37 ± 0.06 B	0.91 ± 0.01 AB	0.91 ± 0.02 B	0.63 ± 0.02 B

^a Values are the means (*n* = 3 experiments) ± 1 standard error. Different letters (A, B, and C) represent significant differences (*P* < 0.05) between values in the same column.

Cu²⁺ concentrations in CCNWSX0020 since SOD and CAT remove excessive oxygen radicals and reduce the toxic effects of reactive oxygen species (ROS). GPX and GR are also protective enzymes of the GSH peroxidase system to handle external oxidative stress, which can directly or indirectly clear intracellular excessive ROS. In the GSH/GPX system, GSH can be oxidized to GSSG by GPX, and GSSG can be reduced to GSH by GR. In addition, thiolates as in GSH can bind and buffer soft metals to reduce free metals in the cell but also protect vital iron-sulfur clusters in various enzymes. So the synthesis, consumption, and the redox state of GSH in *S. meliloti* CCNWSX0020 could play an important role in tolerance to soft metals, such as Cu⁺, Cd²⁺, and Zn²⁺. In addition, *S. meliloti* CCNWSX0020 can produce acidic exopolysaccharides which are required for symbiotic development. Exopolysaccharide can also act as a diffusion barrier against H₂O₂ in *S. meliloti* (42). Additionally, cations can bind to the negatively charged exopolysaccharide on the outside of the cell; therefore, the metal ions are sequestered and do not reach the cytoplasm (43). This could provide additional protection of cells against both oxidative stress and copper toxicity.

The growth and nodulation of host plant inoculated with mutants SXa-1 and SXa-2 were obviously inhibited, and nodulation efficiency was significantly decreased, while the other mutant strains had no effects on the host plant growth and nodulation. The *lpxXL* mutant could not synthesize long-chain fatty acids, which are important components of LPS. LPS is involved in the establishment of a symbiotic relationship between rhizobia and their host plant (44). Therefore, LPS content on the *S. meliloti*

CCNWSX0020 surface was reduced in *lpxXL* mutants, thereby leading to a lower nodulation capability.

Copper accumulations in the shoots and roots of *M. lupulina* were analyzed by inoculation with *S. meliloti* and all mutants. Interestingly, IAA production (13.2 ± 1.1 μg ml⁻¹) of *S. meliloti* CCNWSX0020 was slightly higher than values reported for *S. meliloti* 1021 (11.0 ± 0.8 μg ml⁻¹) and other rhizobia (45). Although IAA production of mutants was less than that of the wild-type strain, compared to the plant biomass inoculated with the wild-type strain, there was no significant effect on dry weight of plants inoculated with the different mutants in the absence of copper ions. However, compared with the biomass of the plant inoculated with the wild-type strain, dry weights of both roots and shoots of plants inoculated with mutants were significantly decreased as the copper concentration increased. The same trend was also found for the amount of copper accumulation in the roots and shoots of *M. lupulina*. The ability of IAA to promote plant growth and increase heavy metal stress tolerance has been reported (46, 47). It seems that IAA produced by *S. meliloti* CCNWSX0020 may indirectly promote metal accumulation by increasing the plant biomass. Moreover, the plant transpiration rate was also affected, which subsequently led to more nutrients and subsequently more energy that was needed for copper detoxification and thereby avoiding lethality by high concentrations of Cu²⁺ in plant tissue (48, 49). In addition to genes necessary for nitrogen fixation, *acdS* (SM0020_23022) encoding ACC deaminase and its upstream regulatory gene (SM0020_23017) were identified in the *S. meliloti* CCNWSX0020 genome. ACC deami-

TABLE 6 Effect of mutants on the copper content (μg/g) in roots and shoots of *M. lupulina* on perlite-vermiculite added with different concentrations of CuSO₄

Strain	Cu ²⁺ content accumulated by roots or shoots of <i>M. lupulina</i> with different treatments ^a			
	Cu ²⁺ content in roots		Cu ²⁺ content in shoots	
	47.36 mg/kg CuSO ₄	142.08 mg/kg CuSO ₄	47.36 mg/kg CuSO ₄	142.08 mg/kg CuSO ₄
Control	75.65 ± 3.42 C	128.96 ± 12.68 C	8.27 ± 0.76 B	13.34 ± 1.77 B
<i>S. meliloti</i> CCNWSX0020	111.15 ± 2.60 A	295.80 ± 1.90 A	12.88 ± 1.40 A	22.03 ± 1.96 A
SXa-1	86.72 ± 2.42 B	131.15 ± 2.47 B	10.24 ± 1.04 AB	16.41 ± 1.72 B
SXa-2	85.38 ± 2.78 B	135.01 ± 3.02 B	10.87 ± 1.36 AB	18.20 ± 1.43 B
SXc-1	81.46 ± 3.18 BC	118.58 ± 3.54 B	9.59 ± 1.09 B	13.71 ± 1.31 B
SXc-2	81.91 ± 3.34 BC	115.58 ± 3.14 B	9.01 ± 1.05 B	14.42 ± 1.95 B
SXn	78.37 ± 1.90 C	170.24 ± 1.40 B	9.06 ± 1.65 B	15.81 ± 1.40 B

^a Values are the means (*n* = 3 experiments) ± 1 standard error. Different superscripts letters (A, B, and C) represent significant differences (*P* < 0.05) between values in the same column.

FIG 6 Alignment of the N-terminal regions of P-type ATPases in *E. coli* and *S. meliloti* CCNWSX0020. The CPx consensus sequence is boxed and the CXXC motif is overlined.

nase can promote the formation of longer roots and lower the level of ethylene to enhance plant survival in contaminated soil (50). *S. meliloti* CCNWSX0020 can produce IAA even under Cu^{2+} -contaminated conditions. An intact potential IAA biosynthesis pathway, the indole-3-acetonitrile (IAN) pathway, was identified in the genome of *S. meliloti* CCNWSX0020. In the IAN pathway, six genes are involved in IAA synthesis. Tryptophan is converted into 5-hydroxytryptamine by aromatic amino acid decarboxylase (SM0020_04045) and then into 3-indole acetonitrile by indole acetaldoxime anhydrase (SM0020_33077). Next, 3-indole acetonitrile is converted into indole-3-acetamide by nitrile hydratase (SM0020_26156, SM0020_26161) and then converted into IAA by amidase (SM0020_03585, SM0020_05557). Trehalose is a nonreducing disaccharide which can stabilize biological structures, including dehydrated enzymes, proteins, and lipids under environmental stress in plants. In the genome of *S. meliloti* CCNWSX0020, three trehalose synthesis pathways, TreY-TreZ, TPS/TPP, and TreS, were identified. The TreS pathway involves the conversion of maltose to trehalose by trehalose synthase (SM0020_21997). In the TreY-TreZ pathway, maltodextrin is first converted to malto-oligosyltrehalose by malto-oligosyltrehalose synthase (SM0020_14699) and then to trehalose by malto-oligosyltrehalose trehalohydrolase (SM0020_04210). In the TPS/TPP pathway, UDP glucose is converted to 6-phosphoric acid trehalose by trehalose 6-phosphate synthetase (SM0020_00020) and then to trehalose by 6-phosphoric acid trehalose phosphatase.

In this study, the *S. meliloti* CCNWSX0020 Tn5 insertion library was created. Five copper-sensitive mutants were isolated, and the genes *lpxXL*, *merR*, and *omp* were disrupted by Tn5. The genome of *S. meliloti* CCNWSX0020 was sequenced, and a number of predicted protein-coding genes, such as *copA*, *cueO*, and *cueR*, involved in copper homeostasis were found. It seems that copper binding proteins (SM0020_14779) can chelate some copper ions when the ion concentration exceeds the normal physiological level. Meanwhile, CueR (SM0020_11410, SM0020_29390) senses cytoplasmic Cu^{+} , and transcription of *copA* (SM0020_11415, SM0020_05727) and *cueO* (SM0020_18797) are activated. Copper chaperones deliver Cu^{+} to CopA, which pumps out excessive copper from the cytoplasm into the periplasm, and CueO oxidizes Cu^{+} to Cu^{2+} in the periplasm, thereby reducing copper toxicity. The negatively charged exopolysaccharide on the outside of the cell could bind and sequester copper ions to prevent them from reaching the cytoplasm. In addition, *S. meliloti* CCNWSX0020 can actively promote host plant growth

through nitrogen fixation, phytohormone, and ACC deaminase. More plant biomass can absorb more copper ions, which subsequently leads to decreasing copper concentration and thereby avoids toxicity to cells.

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