Zinc Resistance Mechanisms of P_{1B}-type ATPases in Sinorhizobium meliloti CCNWSX0020

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The Sinorhizobium meliloti (S. meliloti) strain CCNWSX0020 displayed tolerance to high levels exposures of multiple metals and growth promotion of legume plants grown in metal-contaminated soil. However, the mechanism of metal-resistant strain remains unknown. We used five P_{1B}-ATPases deletions by designating as \textit{\Delta}copA1b, \textit{\Delta}fixI1, \textit{\Delta}copA3, \textit{\Delta}zntA and \textit{\Delta}nia, respectively to investigate the role of P_{1B}-ATPases in heavy metal resistance of S. meliloti. The \textit{\Delta}copA1b and \textit{\Delta}zntA mutants were sensitive to zinc (Zn), cadmium (Cd) and lead (Pb) in different degree, whereas the other mutants had no significant influence on the metal resistance. Moreover, the expression of zntA was induced by Zn, Cd and Pb whereas copA1b was induced by copper (Cu) and silver (Ag). This two deletions could led to the increased intracellular concentrations of Zn, Pb and Cd, but not of Cu. Complementation of \textit{\Delta}copA1b and \textit{\Delta}zntA mutants showed a restoration of tolerance to Zn, Cd and Pb to a certain extent. Taken together, the results suggest an important role of copA1b and zntA in Zn homeostasis and Cd and Pb detoxification in S. meliloti CCNWSX0020.

Heavy and transition metal homeostasis is crucial in all biological systems. Transition metals such as zinc (Zn), copper (Cu), and iron (Fe) are essential micronutrients that are required for many physiological processes but are also extremely toxic in excess. Other metals, such as cadmium (Cd), silver (Ag) and lead (Pb), are acutely toxic and represent a major threat for cell survival. Organisms have evolved to contain multiple defense mechanisms to prevent overaccumulation of heavy and transition metals, such as efflux transport, intracellular sequestration, precipitation, bioadsorption and transformation, etc.

The P_{1B}-type ATPase subfamily belongs to the P-type ATPases family and couples ATP hydrolysis to transition metal transport across cellular membranes. The P_{1B}-type ATPases are the most widely distributed group and have the largest substrate range. P_{1B}-ATPases display several structural characteristics that include six to eight transmembrane helices (TM), the signature sequence (CPC, CPH, SPC, PCP) present in the sixth TM (TM6), one or more metal binding domains in the cytoplasmic N-terminal or C-terminal region (N-MBD, C-MBD) and the catalytic phosphorylation site (DGTGT) between TM6 and TM7. The P_{1B}-ATPases play an essential role in transition metal homeostasis. P_{1B}-ATPases have been divided into five subclasses designated P_{1B-1} to P_{1B-5} according to a combination of substrate specificity, sequence similarity and conserved metal binding residues present in transmembrane segments. The metal specificity of the five P_{1B} ATPase subfamilies have been extensively studied and it has been shown that the P_{1B-1}-ATPases transport Cu\textsuperscript{2+} and Ag\textsuperscript{+}, the P_{1B-2}-ATPases transport Zn\textsuperscript{2+}, Cd\textsuperscript{2+} and Pb\textsuperscript{2+14}, the P_{1B-3}-ATPases are suggested to transport Cu\textsuperscript{2+12} and the P_{1B-4}-ATPases transport Co\textsuperscript{2+}, Ni\textsuperscript{2+} and/or Zn\textsuperscript{2+16}. As to P_{1B-5}, it has been shown that the S. meliloti Sma163 gene encoding for a P_{1B-5}-ATPase that denoted Nia was biochemically characterized indicating Ni\textsuperscript{2+} and Fe\textsuperscript{3+} are substrates of Nia. Moreover, two new subtype of P_{1B}-ATPases (P_{1B-6} and P_{1B-7}) have been recognized recently, but none of these P_{1B-6} and P_{1B-7} ATPases were biochemically examined beyond their sequence classification.

Previous studies on S. meliloti 2011 P_{1B-1}-ATPases showed that five homologous Cu\textsuperscript{2+}-ATPases exhibited functional diversity. These five homologous Cu\textsuperscript{2+}-ATPases are divided into three major subgroups, including CopA1-like transporter (CopA1a and CopA1b), CopA2-like ATPase (FixI1 and FixI2) and CopA3-like ATPase (CopA3). CopA1a is a typical Cu\textsuperscript{2+}-ATPase catalyzing cytoplasmic Cu\textsuperscript{2+} efflux to prevent overaccumulation of cytoplasmic copper. A mutation of copA1a resulted in a copper sensitive phenotype and an increase in
cytoplasmic copper levels. CopA1b displayed 80% homology to CopA1a. However, a mutant of copA1b changed neither copper tolerance nor cytoplasmic copper accumulation. Rather, it changed the differentiation of the mutant strain into bacteroids, the number of viable bacteria (undifferentiated bacteria) in the copA1b mutant strain-induced nodules was increasing faster than differentiated bacteroids. The CopA2-like (FixI) ATPase in *S. meliloti* 2011, is encoded in an operon together with genes encoding cytochrome oxidase subunits. It has been proposed that FixI ATPase may be involved in respiration under microaerobiosis during symbiosis while FixL2 ATPase is required for respiration during all steps of bacterial life. CopA3 looks like a novel Cu⁺-ATPase. Mutation of copA3 gene did not lead to sensitivity to Cu⁺ or cytoplasmic copper accumulation in the mutant strain and the gene was regulated by redox stress and was required during symbiosis. The *P1B*-5 ATPase has not been well characterized. It was shown that *S. meliloti* 2011 Nia was induced by Fe²⁺ and Ni²⁺ and a nia mutant accumulated nickel and iron, suggesting a possible role in Fe²⁺ and Ni²⁺ detoxification.27.

P₁B-2-ATPases (*Zn²⁺/Cd²⁺/Pb²⁺* transporter) are less studied in rhizobia but have been well characterized in other bacteria such as ZnTα in *Escherichia coli* and CadA in *Staphylococcus aureus*. ZnTα is not only for *Zn²⁺* efflux but also transports the non-physiological substrates Cd²⁺ and Pb²⁺.12. The expression of the zntA gene is activated via the Zn²⁺-responsive transcriptional regulator (ZntR)20. CadA is known to encode a Cd²⁺ efflux ATPase which plays a role in the cadmium resistance of *S. aureus*21. In *S. meliloti* 1021 the SMc04128 gene encodes a P₁B-2-type ATPase. A transposon insertion mutant of SMc04128 showed sensitivity to high concentrations of *Zn²⁺* and *Cd²⁺* and slightly increased sensitivities to *Cu²⁺*, *Pb²⁺*, *Ni²⁺*, and Co²⁺, which indicated that SMc04128 plays a role in the defense of *S. meliloti* 1021 against these heavy metals.22 Moreover, it has been reported that a gene named *cadA* in *Mesorhizobium metallidurans* isolated from a zinc-rich mining soil also encodes a P₁B-2-type ATPase involved in cadmium and zinc resistance. The *cadA* gene was induced by zinc and cadmium and a *cadA*-deleted strain failed to grow at high zinc and cadmium concentrations.23

*Sinorhizobium meliloti* CCNWSX0020 was isolated from *Medicago lupulina* growing in gold mine tailings in the northwest of China and exhibited higher tolerance towards multiple metals, such as Cu, Zn, Cd and Pb. The heavy metal transporting P₁B-type ATPases in *S. meliloti* CCNWSX0020 remains unknown. There are five genes encoding putative P₁B-type ATPases on the *S. meliloti* CCNWSX0020 genome. Their predicted signature transmembrane metal binding residues indicated that three genes encode three P₁B-1-ATPase, one (SM0020_22747) encoded a P₁B-2-ATPase, another (SM0020_05862) encoded a putative P₁B-3-ATPase. These five P₁B-ATPases are predicted to be mainly responsible for heavy metals homeostasis and detoxification in *S. meliloti* CCNWSX0020. To further investigate the function of these five P₁B-ATPases we created deletions in these genes and tested different metal tolerance of these deletions. Both deletion of SM0020_11415 (*Cu²⁺*-ATPase) and deletion of SM0020_22747 (*Zn²⁺*-ATPase) displayed sensitivity to *Zn²⁺*, *Cd²⁺* and *Pb²⁺*. To test whether SM0020_11415 and SM0020_22747 in *S. meliloti* CCNWSX0020 have similar functions, we investigated these genes expressions in response to different levels of heavy metals exposure and their capability to complement the *ΔcopA* and Δ*zntA* E. coli mutant strains. The combined results of these studies suggest that SM0020_22747 encodes a classical *Zn²⁺*-ATPase which is required for efflux of *Zn²⁺*, *Cd²⁺* and *Pb²⁺*, whereas SM0020_11415 encoding a *Cu²⁺*-ATPase surprisingly confers tolerance to *Zn*, *Cd* and *Pb* but not to *Cu* in *S. meliloti* CCNWSX0020.

**Results**

**Deletion of P₁B-type ATPases made mutant strains more sensitive to a number of heavy metals.**

Bioinformatics studies have shown that the genomes of many bacteria including *S. meliloti* contain a diverse array of genes encoding a number of P₁B-ATPases. P₁B-ATPases have been associated with the detoxification and tolerance mechanism of heavy and transition metals. *S. meliloti* CCNWSX0020, could tolerate up to 1.4 mM CuSO₄, 1.0 mM ZnSO₄, 3.2 mM Pb(NO₃)₂, 0.25 mM CdSO₄ and 1.0 mM NiSO₄ in TY solid medium. A phylogenetic analysis of five predicted P₁B-type ATPases from *S. meliloti* CCNWSX0020 indicated three of them were Cu⁺-ATPases, one was Zn²⁺-ATPase and the last belongs to P₁B-type ATPase (Fig. 1). Furthermore, the results of sequence homology analysis, the three Cu⁺-ATPases genes (SM0020_05727, SM0020_05912 and SM0020_11415) displayed the highest similarity with the *copA3*, *fixI1*, and *copA1b* genes which were previously identified in *S. meliloti* 201119. The SM0020_22747 gene was 99.1% identical to the zntA gene of *S. meliloti* 1021 while SM0020_05862 showed 98% similarity to the *nia* gene that encoded a nickel (Ni) and Fe transporter in *S. meliloti* 201117,22. We therefore selected all five P₁B-ATPases that involved in heavy metals resistance of *S. meliloti* CCNWSX0020. To test our hypotheses, the five P₁B-ATPase deletions (*ΔcopA1b*, *ΔcopA3*, *ΔfixI1*, Δ*zntA*, and Δ*nia*) were characterized using metal-tolerance growth assays in TY liquid medium. The wild type strain and five deletion mutants were cultured in TY supplemented with increasing concentration of CuSO₄, ZnSO₄, CdSO₄, Pb(NO₃)₂, and NiCl₂ (Fig. 2). The Δ*zntA* mutant showed the greatest sensitivities to 0.2 mM *Zn²⁺* and 0.05 mM *Cd²⁺*, while *ΔcopA1b* mutant was slightly more sensitive to high concentration of *Zn²⁺* (0.6 mM) and *Cd²⁺* (0.15 mM) (Fig. 2A–B). Both Δ*zntA* and Δ*copA1b* mutants showed sensitivity to high concentration of *Pb²⁺* in different degrees and had no effect on Cu and Ni tolerance (Fig. 2C–E). The CuSO₄, ZnSO₄, CdSO₄, Pb(NO₃)₂, and NiCl₂ metals tolerance of the other three mutants (*ΔcopA3*, *ΔfixI1*, and Δ*nia*) displayed no difference to the wild type strain (Fig. 2). These results suggested *CopA1b* and ZntA were involved in Zn, Cd and Pb metals resistance in *S. meliloti* CCNWSX0020. To further explore the function of *CopA1b* and ZntA, these two zinc sensitive deletions were further studied.

**CopA1b and ZntA could play a role in zinc, cadmium and lead homeostasis.**

The *ΔcopA1b* and *ΔzntA* mutants of *S. meliloti* CCNWSX0020 exhibited sensitivity to *Zn²⁺*, *Cd²⁺* and *Pb²⁺* but not to other metals, suggesting *CopA1b* and ZntA may play a role in Zn, Cd, and Pb homeostasis in this strain. To verify the presence of the respective *copA1b* and *zntA* genes or either gene alone that was responsible for Zn, Cd, and Pb resistance,
the two genes were amplified and inserted into the pBRR1MCS-5 vector and transformed into the corresponding mutant and then tested for these metals tolerance. Figure 3 shows the complemented strains (C-\(\text{copA1b}\) and C-\(\text{zntA}\)) could restore Zn, Cd and Pb resistance of the mutants by 80%. These results demonstrated that the sensitivity of three metals to the mutants was due to the deletion of \(\text{copA1b}\) and \(\text{zntA}\) in \(\text{Sinorhizobium meliloti}\) CCNWSX0020.

Figure 1. Phylogenetic analysis of P\(_{1B}\)-type ATPases. Branches indicating proteins in subgroups IB-1, IB-2, IB-3, IB-4 and IB-5 are under different colors. Five P\(_{1B}\)-type ATPases genes (SM0020_05727/\(\text{copA3}\), SM0020_05862/\(\text{nix}\), SM0020_05912/\(\text{fixi}\), SM0020_11415/\(\text{copA1b}\), and SM0020_22747/\(\text{zntA}\)) in \(\text{Sinorhizobium meliloti}\) CCNWSX0020 are tagged in the unrooted tree.

Figure 2. Influence of deletions in genes encoding different P\(_{1B}\)-type ATPases on metal tolerance of \(\text{Sinorhizobium meliloti}\) CCNWSX0020. Wild type and mutant strains were grown in TY liquid medium for 48 h in the presence of increasing concentrations of zinc (A), cadmium (B), lead (C), copper (D), and nickel (E). Symbols represent the wild type strain (WT) and mutants ∆\(\text{copA3}\), ∆\(\text{nix}\), ∆\(\text{fixi}\), ∆\(\text{copA1b}\), and ∆\(\text{zntA}\) of \(\text{S. meliloti}\) CCNWSX0020 (●, ■, ▲, ×, *, ●, respectively). Error bars represent standard deviations of three biological repeats.
Thus we can speculate that copA1b and zntA genes are involved in Zn homeostasis and Cd or Pb detoxification. Surprisingly zntA encodes a Zn\(^{2+}\)/Cd\(^{2+}\)/Pb\(^{2+}\) transporter whereas copA1b is predicted to encode a Cu\(^{+}\)/Ag\(^{+}\) transporter. A mutant containing a zntA deletion did not grow in the presence of low Zn levels as expected. In contrast, the growth of a mutant containing a copA1b deletion was not inhibited by high Cu levels but rather by high concentrations of Zn, Cd and Pb. To better understand the differences between the two genes in responsive to metals exposure, the capabilities of S. meliloti CopA1b and ZntA to complement the E. coli ΔcopA and ΔzntA strains were tested. As expected, CopA1b could complement the Cu sensitive phenotype of E. coli ΔcopA strain while ZntA could complement the Zn sensitive phenotype of E. coli ΔzntA strain. Meanwhile, CopA1b could restore Zn tolerance of an E. coli ΔzntA strain to some degree whereas ZntA failed to restore Cu tolerance of an E. coli ΔcopA strain (Fig. 4). These results suggested that CopA1b and ZntA conferred resistance to Zn, Cd and Pb tolerance and CopA1b also had a capacity for Cu tolerance.

Figure 3. Growth in TY solid media of wild type, mutant strains (ΔcopA1b and ΔzntA) and complemented strains (C-copA1b and C-zntA) of S. meliloti CCNWSX0020. Five 10-fold dilutions were spotted from left to right, in the presence of the indicated concentrations of ZnSO\(_4\), CdSO\(_4\) and Pb(NO\(_3\))\(_2\).

Figure 4. Complementation of ΔcopA E. coli copper sensitive phenotype and ΔzntA E. coli zinc sensitive phenotype by heterologously expressed S. meliloti Cu\(^{+}\)-ATPase (C-copA1b) and Zn\(^{2+}\)-ATPase (C-zntA). All strains were grown to exponential phase in LB liquid medium. Five 10-fold dilutions were carried out and spotted on the LB agar plates from left to right, in the presence of the indicated concentrations of CuSO\(_4\) and ZnSO\(_4\).
Expression of copA1b and zntA could be induced by different types of heavy metals. To further investigate CopA1b and ZntA in *S. meliloti* CCNWSX0020, the gene expressions of zntA and copA1b under different metal stresses were examined using qRT-PCR. The expression profiles of copA1b and zntA genes in wild type, ∆copA1b, and ∆zntA mutants of *S. meliloti* CCNWSX0020 strains at OD600 of 1.0 were incubated with 0.6 mM CuSO4, 0.05 mM AgNO3, 0.4 mM ZnSO4, 0.1 mM CdSO4 and 1.5 mM Pb(NO3)2 for 30 min. Samples were then processed for qPCR analysis and normalized against the ribosomal 16S rRNA. Error bars represent standard deviations of three biological repeats. **P < 0.01.

Figure 5. Gene expression analysis. Expression of copA1b (SM0020_11415) and zntA (SM0020_22747) under copper, silver, zinc, cadmium and lead stress (A, B). (C) Expression of genes in the vicinity of copA1b (SM0020-11415) under copper, silver and zinc stress. Wild type, ∆copA1b, and ∆zntA mutants of *S. meliloti* CCNWSX0020 strains at OD600 of 1.0 were incubated with 0.6 mM CuSO4, 0.05 mM AgNO3, 0.4 mM ZnSO4, 0.1 mM CdSO4 and 1.5 mM Pb(NO3)2 for 30 min. Samples were then processed for qPCR analysis and normalized against the ribosomal 16S rRNA. Error bars represent standard deviations of three biological repeats. **P < 0.01.
High levels of intracellular Cu. This result demonstrated that CopA1b was not involved in Cu export and (AAS). The cells were grown in TY medium individually supplemented with 0.4 mM ZnSO4, 2.0 mM Pb(NO3)2, internal metal content of wild type and mutant cells were measured by furnace atomic absorption spectroscopy that CopA1b and ZntA play roles in expelling these surplus metals from cytoplasm. To test this hypothesis, total Zn, Cd and Pb is that higher levels of these metal ions were accumulated in the mutant cells. This would imply whereas the enzyme activities showed no major differences under H2O2-free condition (Fig. 7A,B). The total SOD activity of S. meliloti by atomic absorption spectrophotometer. Error bars represent standard deviations of three biological repeats. *P < 0.05, ***P < 0.001.

Deletions of copA1b and zntA led to increased intracellular concentrations of zinc, lead and cadmium but not copper. A possible explanation for the sensitivity of ΔcopA1b and ΔzntA mutants toward Zn, Cd and Pb is that higher levels of these metal ions were accumulated in the mutant cells. This would imply that CopA1b and ZntA play roles in expelling these surplus metals from cytoplasm. To test this hypothesis, total internal metal content of wild type and mutant cells were measured by furnace atomic absorption spectroscopy (AAS). The cells were grown in TY individually supplemented with 0.4 mM ZnSO4, 2.0 mM Pb(NO3)2, 0.05 mM CdSO4 and 0.8 mM CuSO4. The ΔzntA mutant which was hypersensitive to Zn and Cd accrued significantly (P < 0.01) higher amounts of intracellular Zn (~3 fold), Cd (~8 fold) and Pb (~2.5 fold) compared with the WT (Fig. 6). The ΔcopA1b mutant had a smaller but significant effect on the metal content since the loss of copA1b led to an increased accumulation of Zn by 1 fold, Cd by 1.5 fold (P < 0.05) and Pb by 2 fold (P < 0.01) relative to the WT (Fig. 6). The increase in the accumulation of Zn, Cd and Pb in the ΔcopA1b and ΔzntA mutants suggested that CopA1b and ZntA play a role in the efflux of Zn2+ and Cd2+ ions. In addition, the accumulation of intracellular Cu was quite high in the ΔcopA1b and ΔzntA mutants as well as in the WT strain (Fig. 6). This result demonstrated that CopA1b was not involved in Cu export and S. meliloti CCNWSX0020 accumulates high levels of intracellular Cu.

Deletions of copA1b and zntA decreased antioxidant enzyme activity. P1B-ATPases also have a function in providing metals for assembly of periplasmic metalloproteins since some heavy and transition metals are essential component of many free-radical detoxifying enzymes, like catalase, peroxidase and superoxide dismutase. So the capability of ΔcopA1b and ΔzntA to cope with redox stress was tested. Although the H2O2 resistance on the agar plates of ΔcopA1b and ΔzntA mutants showed no big change with S. meliloti wild type strain (data not shown), the CAT, POD and total SOD activities were dramatically changed. When all three strains were treated with H2O2, the CAT and POD activities increased obviously while the total SOD activity displayed no change (Fig. 7). However, compared to S. meliloti wild type strain, the CAT and POD activity levels of ΔcopA1b and ΔzntA mutants showed apparent decline to a different degree under H2O2 treatment condition whereas the enzyme activities showed no major differences under H2O2-free condition (Fig. 7A,B). The total SOD activity of ΔcopA1b and ΔzntA mutants also decreased slightly in comparison to a wild type strain whether under H2O2 treatment or not (Fig. 7C). These results suggest that deletions of CopA1b and ZntA have an influence on the activity levels of these antioxidant enzymes.

Discussion
Sinorhizobium meliloti CCNWSX0020 is a multiple heavy metals resistant bacterium isolated from root nodules of M. lupulina growing on mine tailings in the northwest of China. The genome of S. meliloti CCNWSX0020 has been sequenced and some copper resistance genes have been analyzed in the previous studies. It has been reported that merR encoding an MerR family transcriptional regulator displayed significantly decreased copper resistance in a merR-interrupted mutant of S. meliloti CCNWSX0020 and the expression of two genes (SM0020_11420 and SM0020_11425) encoding putative P1B-type ATPases were found to be down-regulated under Cu/Zn2+/Pb2+/Cd2+ stresses in this merR mutant. These results suggested that the P1B-type ATPases might be involved in heavy metal resistance of S. meliloti CCNWSX0020. To test this hypothesis, the five P1B-type ATPases in S. meliloti CCNWSX0020 were further studied. The phylogenetic analysis of these P1B-type ATPases
suggested that three of them are predicted to be Cu\(^{2+}\)-ATPase, one to be a Zn\(^{2+}\)-ATPase and the last one to be a P\(_{\text{IB}}\)-type ATPase which may be a Ni\(^{2+}\)-ATPase.

According to the analysis of deletion mutants the five genes encoding P\(_{\text{IB}}\)-type ATPases obtained upon double homologous recombination, a ΔzntA mutant was shown to be hypersensitive to low concentrations of Zn and Cd compared to other mutants and the wild type, indicating the vital role of ZntA in Zn and Cd resistance in *S. meliloti* CCNWSX0020. As pointed out in previous studies, ZntA in *S. meliloti* CCNWSX0020 contains a conserved phosphorylation motif (DKTGT), a CXXC (CASC) motif in N terminal and typical conserved residues of P\(_{\text{IB}}\)-ATPases in trans-membrane helices including a CPC motif in TM6, a T(X)\(_4\)Q(N)(X)K motif in TM7 and a DXG(X)\(_7\)N motif in TM8 (see Supplementary Fig. S1)\(^9,34\). The well-characterized ZntA from *E. coli* mediates the efflux of Zn\(^{2+}\), Cd\(^{2+}\) and Pb\(^{2+}\)\(^13\). Amino acid alignment of ZntA from *S. meliloti* CCNWSX0020 showed 98.04% identity to the Smc04128-encoded P\(_{\text{IB}}\)-type ATPase in *S. melliloti* 1021 which plays a crucial role in the defense of *S. melliloti* against high concentrations of Zn and Cd\(^2\). The *S. meliloti* CCNWSX0020 ΔzntA mutant is highly sensitive to low concentration of Zn and Cd but only slightly sensitive to high concentration of Pb, suggesting that ZntA in *S. meliloti* CCNWSX0020 is essential to the resistance against these metal ions. RT-PCR showed that the expression of zntA in *S. meliloti* CCNWSX0020 was induced by heavy metals with the following order of effectiveness: Cd\(^{2+}\) > Zn\(^{2+}\) > Pb\(^{2+}\). In addition, a *S. meliloti* CCNWSX0020 ΔzntA mutant displayed an increased intracellular accumulation of Zn, Pb and Cd. These results strongly suggest ZntA in *S. meliloti* CCNWSX0020 to be a typical Zn\(^{2+}\)-ATPase having a crucial role in the efflux of Zn, Cd and Pb.

Previous studies have reported the presence of five Cu\(^{2+}\)-ATPase genes on the *S. melliloti* 2011 genome and analyzed the functional diversity of these five homologous Cu\(^{2+}\)-ATPases\(^19\). The authors divided these five Cu\(^{2+}\)-ATPases into three subgroups including CopA1-like ATPases (CopA1a and CopA1b), CopA2-like ATPases (FixI1 and FixI2) and CopA3-like ATPases. Based on sequence alignment, three genes (*SM0020_05727, SM0020_05912* and *SM0020_11415*) encoding Cu\(^{2+}\)-ATPases on the *S. melliloti* CCNWSX0020 genome were very similar to CopA3, FixI1 and CopA1b, respectively. The deletions of putative Cu\(^{2+}\)-ATPases (*ΔcopA1b, ΔcopA3* and *ΔfixI1*) had no effect on Cu resistance in agreement with results obtained from mutants of the genes encoding homologous Cu\(^{2+}\)-ATPases in *S. melliloti* 2011. However, one important difference from *S. melliloti* 2011 was that a *ΔcopA1b* deletion in *S. melliloti* CCNWSX0020 displayed sensitivity to high concentrations of Zn, Cd and Pb. In addition, we could not identify a typical Cu\(^{2+}\)-translocating P\(_{\text{IB}}\)-ATPase such as CopA1a in *S. melliloti* 2011 on the genome of *S. melliloti* CCNWSX0020. Moreover, cells of *S. melliloti* CCNWSX0020 accumulated high amounts of Cu in both the wild type strain and the different mutant strains. This indicate that copper resistance in *S. melliloti* CCNWSX0020 is not due to efflux but rather increased copper binding in cells most likely in the periplasm.

Based on sequence alignment, *S. melliloti* CCNWSX0020 CopA1b was predicted to be a Cu\(^{2+}\)-translocating P\(_{\text{IB}}\)-ATPase and CueR located upstream of *copA1b* was responsible for Cu and Ag-dependent induction of *copA1b* expression. Moreover, *copA1b* could confer copper tolerance to a copper sensitive *E. coli* ΔcopA strain (Fig. 4). These results suggested that CopA1b itself has a capability for copper tolerance and/or efflux. However, ΔcopA1b mutant in *S. melliloti* CCNWSX0020 did not lead to copper sensitivity or increased copper accumulation. Perhaps the lack of copper sensitive phenotype in the ΔcopA1b mutant was masked by functional redundancy with other copper transporters or copper resistance determinants. Based on a previous study in *S. melliloti* CCNWSX0020, some genes responsible for copper homeostasis could be identified. These genes include *lpxXL* (*SM0020_18047*) encoding the LpxXL C-28 acyltransferase, *omp* (*SM0020_18792*) encoding a hypothetical outer membrane protein, *cueO* (*SM0020_18797*) encoding a periplasmic multicopper oxidase, and *merR* (*SM0020_29390*) encoding a regulatory activator\(^32\). Therefore, we compared expression of these four genes with expression of the genes encoding these three Cu\(^{2+}\)-ATPase (*SM0020_05727/copA3, SM0020_05912/fixI1, SM0020_11415/copA1b*) under Cu stress. The data showed that *omp* and *cueO* were highly induced by Cu to
trations, demonstrating that although $\text{CopA1b}$ might be responsible for delivery of Cu into metalloenzymes (such as Cu/Zn-SOD) that protect against metal or oxidative stress. In line with previous work demonstrating that although $\text{CopA1b}$ and $\text{ZntA}$ are potentially being involved in assembly and maturation of metalloenzymes crucial for copper resistance mechanism of $S. \text{meliloti}$ (data not shown).

The $\Delta \text{copA1b}$ mutant was sensitive to Zn, Cd and Pb, and led to increased intracellular Zn, Cd and Pb concentrations, demonstrating that although $\text{copA1b}$ in $S. \text{meliloti}$ is predicted to encode a Cu $\text{ATPase}$, it is involved in Zn, Cd and Pb homeostasis. Previous studies have reported that P$_{1B}$-ATPases have a high specificity for substrate they transport, for example Cu $\text{ATPase}$ transports monovalent heavy metal ions (Cu$^{+}$, Ag$^{+}$) and Zn$^{2+}$-ATPase transports divalent heavy metal ions (Zn$^{2+}$, Cd$^{2+}$)$^{39,40}$. Sequence comparisons and functional characterization have underlined the importance of the difference between Cu $\text{ATPases}$ and Zn$^{2+}$-ATPases in the presence of unique and conserved trans-membrane amino acid residues which could contribute to substrate specificity, such as Tyr/Asn of TM7, Pro/Met/Ser of TM8 in Cu $\text{ATPase}$ while Leu/Val/Ala of TM8 in Zn$^{2+}$-ATPase$^{34-36}$. Furthermore, in agreement with most Cu $\text{ATPases}$, $\text{CopA1b}$ in $S. \text{meliloti}$ CCNWSX0020 contains two CXXC(CASC) motifs in the N terminal and typical conserved residues of P$_{1B}$-ATPases in trans-membrane helices including a CPC motif in TM6, a YN(X)P motif in TM7, a MXSS motif in TM8 (see Supplementary Fig. S1)$^{33,34}$. It is probable that the presence of the same N-terminal CASC motif in $\text{CopA1b}$ and $\text{ZntA}$ in $S. \text{meliloti}$ CCNWSX0020 could bind Zn$^{2+}$/Cd$^{2+}$/Pb$^{2+}$ ions. However, the function of the N-terminal CXXC motif in P$_{1B}$-type $\text{ATPase}$ remains controversial, as two N-terminal CXXC motifs in E. coli $\text{CopA}$ have distinct functions (the MBD1 functions as metallochaperones and the MBD2 has a regulatory role in CopA activity)$^{33}$ while CXXC motif in Streptococcus pneumoniae $\text{CopA}$ is able to bind a dicopper center and might be responsible for delivery of Cu$^{2+}$ to the TM metal-binding sites$^{38}$. To study the function of N-terminal CXXC motif of $\text{CopA1b}$ and $\text{ZntA}$ in $S. \text{meliloti}$ CCNWSX0020, we constructed these two N-terminal deletions. The results showed that the zntA $\text{N-terminal deletion}$ was slightly sensitive to high zinc concentration while $\text{copA1b}$ N-terminal deletion did not affect copper and zinc tolerance in $S. \text{meliloti}$, and that neither N-terminal deletions of $\text{CopA1b}$ nor $\text{ZntA}$ could restore copper or zinc tolerance of E. coli $\Delta \text{copA}$ and $\Delta \text{zntA}$ mutant strains (data not shown). These indicate that the presence of N-terminal domain of $\text{CopA1b}$ and $\text{ZntA}$ is essential for their complete transportation function. In addition, the $S. \text{meliloti}$ $\text{CopA1b}$ confers a slight increase in zinc tolerance of E. coli $\Delta \text{zntA}$ mutant (Fig. 4). Therefore, it is very possible that the similar N-terminal domain of CopA1b to ZntA could bind Zn$^{2+}$/Cd$^{2+}$/Pb$^{2+}$ ions but these metal ions binding by CopA1b N-terminal domain is not essential for cellular Zn$^{2+}$/Cd$^{2+}$/Pb$^{2+}$ resistance, just played a primary role in cytoplasmic Zn$^{2+}$/Cd$^{2+}$/Pb$^{2+}$ sequestration or delivery to the transmembrane site of CopA1b for cellular efflux. Notably, similar to $\text{copA1b}$, SMc04128 gene in S. meliloti 1021 encoded a Zn$^{2+}$-ATPase and transposon insertion mutant of SMc04128 was not only highly sensitive to Zn$^{2+}$ and Cd$^{2+}$ but also slightly increased sensitivities to Cu$^{2+}$, Pb$^{2+}$, Ni$^{2+}$ and Co$^{2+}$.$^{22}$ This phenomenon has attracted our attention and it is likely that Cu$^{2+}$-ATPase and Zn$^{2+}$-ATPase in S. meliloti might not have strict specificity for the heavy and transition metal ions they transport.

Moreover, P$_{1B}$-ATPases were also shown to be involved in protein maturation and delivering metal cofactors into metalloenzymes$^{39,40}$. Previous work demonstrated that both functions are important for bacterial virulence$^{25,54}$. A novel host immune defense against bacterial invaders was identified and involved intoxication by transition metals (such as copper and zinc) in the phagosome to kill bacteria$^{42-44}$. This mechanism could also be identified in response to protozoa$^{46}$. P$_{1B}$-ATPases in bacteria are required for transition metal efflux and assembly of metalloproteins which are essential for bacterial survival in extreme oxidative environments$^{46}$. So we speculate that although CopA1b in S. meliloti CCNWSX0020 is not responsible for copper tolerance, it may incorporate copper into metalloenzymes (such as Cu/Zn-SOD) that protect against metal or oxidative stress. In line with our speculation, the deletion of $\text{copA1b}$ led to a decrease in CAT, POD and total SOD activity level (Fig. 7), so a $\text{copA1b}$ deletion could result in sensitivity to high concentration of Zn, since $\Delta \text{copA1b}$ mutant might lack enough capability to cope with the oxidative stress caused by high Zn. However, Cu and Zn usually served as cofactor for Cu/Zn-SOD not for CAT and POD, but the deletions of $\text{copA1b}$ and $\text{zntA}$ also decreased the CAT and POD activity levels. It is speculated that the reduced CAT and POD activity might result from the periplasmic metal disturbances caused by deletions of $\text{copA1b}$ and $\text{zntA}$.

In conclusion, in S. meliloti CCNWSX0020 ZntA is a typical Zn$^{2+}$-ATPase playing a crucial role in the efflux of Zn, Cd, and Pb while $\text{copA1b}$ encoding a Cu$^{2+}$-ATPase is involved in tolerance to Zn, Cd, and Pb. Moreover, both of CopA1b and ZntA are potentially being involved in assembly and maturation of metalloenzymes crucial for tolerance to heavy metal and oxidative stress.

Materials and Methods

Bacterial strains, plasmids and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli strains (E. coli DH5α, E. coli GR16 and E. coli RW3110) were grown in Luria-Bertani (LB) medium at 37 °C. Sinorhizobium meliloti CCNWSX0020 was grown at 28 °C in TY medium (5 g tryptone, 3 g yeast extract, and 0.7 g CaCl$_2\cdot$2 H$_2$O per liter)$^{37}$. Media were supplemented with the following antibiotics as required: 100 μg/mL ampicillin (Amp), 50 μg/mL kanamycin (Km), 100 μg/mL gentamicin (Gm) (Table 1).

Bioinformatic analysis. The known P$_{1B}$-ATPase protein sequences of most bacterial genomes used in our study were obtained from UniProtKB (http://www.uniprot.org/uniprot)$^{48}$. The whole set of bacterial P$_{1B}$-ATPase sequences were aligned using ClustalW2$^{49}$ and the phylogenetic tree visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). The neighboring genes of the genes encoding P$_{1B}$-ATPases were obtained from the draft genome sequence of S. meliloti CCNWSX0020 which had been reported with the accession number AGVV00000000.1 in GenBank$^{24}$.
Generation of deletion mutants in genes encoding \( P_{138} \)-type ATPases. An in-frame, tagged \( P_{138} \)-ATPase deletion mutant of \( S. meliloti \) was constructed by a method involving crossover PCR and the suicide vector pK18mobsacB, which cannot replicate in \( S. meliloti \). The total genomic DNA of \( S. meliloti \) was extracted according to the protocol of Wilson and Carson. The plasmid pK18mobsacB-\( \Delta copA1b \) was used to construct the \( S. meliloti \) deletion mutant. A 683 bp upstream and a 655 bp downstream fragment of \( copA1b \) were amplified using primer pairs \( copA1b-F1/copA1b-R1 \) and \( copA1b-F2/copA1b-R2 \), respectively. The upstream and downstream PCR products were ligated by crossover PCR with primer pairs \( copA1b-F1/copA1b-R2 \). The resulting fragment was cloned into pMD18-T plasmid and 

| Strains or plasmids | Relevant characteristics | Source or reference |
|---------------------|-------------------------|--------------------|
| pK18mobsacB         | Suicide vector derived from plasmid pK18, Mob⁺ sacB Km’ | 51 |
| pBBR1MCS-5          | Broad-host-range cloning vector, Gm’ | 60 |
| pRK2013             | Broad-host-range helper vector, Tra⁺ Km’ | University of York, UK, Tanya Soule |
| pMD18-T easy        | Cloning and sequencing vector, Amp’ | TaKaRa |

**Table 1.** Bacterial strains and plasmids used in this study.

Heavy and transition metal sensitivity assays of the five mutants. *Sinorhizobium meliloti* CCNWSX0020 and each of the five deletion mutants were grown to midexponential phase in TY liquid medium at 28°C with shaking at 150 rpm and cell suspensions were prepared at the same \( OD_{600} \) of 1.0 (optical density at 600 nm). Then 1% of the cell suspensions were added to fresh TY medium supplemented with different concentrations of CuSO₄, ZnSO₄, CdSO₄, NiCl₂, and Pb(NO₃)₂. The cells were again incubated with shaking at 150 rpm for 48 h, and growth was monitored at \( OD_{600} \). The data were shown as the means of biological triplicates ± SD.

**Complementation of \( \Delta copA1b \) and \( \Delta zntA \) mutants.** To complement the \( copA1b \) mutant, the entire \( copA1b \) gene including the regulatory region was amplified with primers C-\( copA1b \)-F/C-\( copA1b \)-R using \( S. meliloti \) CCNWSX0020 genomic DNA as template. The PCR product was digested with Smal/XbaI and inserted into broad-range plasmid pBBR1MCS-5 to generate pBBR-\( copA1b \). The sequence of this construct to be used.
in complementation was verified by automated DNA sequencing, transformed into E. coli donor strain DH5α, and delivered into the ΔcopA1b mutant via triparental conjugation. Single clones carrying pBBR-copA1b were selected on TY plates containing gentamicin. The presence of the copA1b gene in the mutant strain was confirmed by PCR. The complementation of a zntA deletion was performed in a similar fashion.

Metal sensitivity assays of S. meliloti CCNWSX0020 wild type, ΔcopA1b mutant and ΔzntA mutant and the corresponding complementations (C-copA1b and C-zntA) were performed on TY solid medium with different concentrations of CuSO4, ZnSO4, CdSO4, and Pb(NO3)2. Cells were grown to exponential phase in TY liquid medium and then diluted to an OD600 of 0.1. Four 10-fold dilutions were carried out and spotted on the agar medium. Each experiment was repeated three times.

Heterologous expression of S. meliloti copA1b and zntA in E. coli ΔcopA and ΔzntA strains. Complementation plasmids pBBR-copA1b and pBBR-zntA were transformed into E. coli ΔcopA strain and E. coli ΔzntA strain via triparental conjugation, respectively. Single clones carrying pBBR-copA1b or pBBR-zntA were selected on LB plates containing gentamicin. The presence of the copA1b or zntA gene in the E. coli ΔcopA and ΔzntA strains was confirmed by PCR.

Copper and zinc sensitivity assays of E. coli ΔcopA and ΔzntA strains and the corresponding complementation (C-copA1b-EΔcopA, C-copA1b-EΔzntA, C-zntA-EΔcopA and C-zntA-EΔzntA) were performed on LB solid medium. Cells were grown to exponential phase at 37°C in LB liquid medium and then diluted to an OD600 of 0.1. Five 10-fold dilutions were carried out and spotted on the agar medium with indicated concentrations of CuSO4 and ZnSO4. Each experiment was repeated three times.

Heavy and transition metal accumulation assay. Sinorhizobium meliloti CCNWSX0020 wild type and mutant strains were grown at 28°C in TY liquid medium until cells reached exponential phase at the same OD600 of 0.8. Then cells were incubated for another 24 h with shaking at 150 rpm after TY medium had been supplemented with 0.8 mM CuSO4, 0.4 mM ZnSO4, 0.05 mM CdSO4 and 2.0 mM Pb(NO3)2. Then cells were harvested by centrifugation at 8000 g for 10 min. The intracellular accumulated heavy and transition metals were measured by furnace atomic absorption spectroscopy (Varian SpectrAA 880/GTA 100) as described previously. The data were shown as the means of biological triplicates ± SD.

H2O2 sensitivity test and antioxidant enzyme activity assay. Sinorhizobium meliloti CCNWSX0020 wild type strain, ΔcopA1b and ΔzntA mutants were grown to exponential phase in TY liquid medium and then diluted to an OD600 of 0.1. Four 10-fold dilutions were carried out and spotted on the agar medium with different concentration of H2O2 for H2O2 sensitivity test.

The exponential phase cells grown in TY liquid medium were treated or untreated with 500 μM H2O2 for 30 min. Then cells were harvested by centrifugation at 8000 g for 2 min and resuspended with enzyme extracting solution. Cell suspensions were lysed by ultrasonic disruption, followed by centrifugation at 8000 g for 10 min. Clear lysates were used for total protein determination and catalase, peroxidase and superoxide dismutase activity assay. Protein concentrations were determined by using the Bradford Bio-Rad protein assay. The catalase (CAT) activity was assayed by measurement of the degradation of H2O2 at a wavelength of 240 nm according to the method of Hammerschmidt, Nuckles and Kuc. The superoxide dismutase (SOD) activity was assayed by measuring the enzyme’s ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described previously. The data were shown as the means of biological triplicates ± SD.

Real-time qRT-PCR analysis. Sinorhizobium meliloti CCNWSX0020 and two zinc-sensitive mutants grown to exponential phase in TY liquid medium were supplemented with 0.6 mM CuSO4, 0.05 mM AgNO3, 0.4 mM ZnSO4, 0.1 mM CdSO4 and 1.5 mM Pb(NO3)2 and incubated for 30 min at 28°C. Then cells were harvested and total RNA was extracted. Procedures including DNA elimination, cDNA synthesis and quantitative RT-PCR were performed as described previously. All these assays were performed in triplicate. Primer pairs used to monitor transcription of genes are listed in Table S1. To standardize results, 16S rRNA was used as an internal standard and the relative levels of transcription were calculated using the 2−ΔΔCT method.

Statistical analyses. Statistical analyses were carried out using SPSS 19.0 software (SPSS 208 Inc., Chicago, IL, USA). Paired two-tailed Student’s t-test was performed to determine significant differences among the different experimental treatments. All of the data was analyzed using the Origin Pro v8.0 (Origin Lab, Hampton, USA) to create the figures.

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Author Contributions
G.W. and Z.L. conceived and designed the experiment, M.L., J.L. and Y.W. performed experiments. M.L. wrote the manuscript and C.R. revised the manuscript and assisted with manuscript preparation. All authors have read the manuscript and agree with its content.

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