Environmental Science & Technology

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Copper Tolerance Mechanisms of *Mesorhizobium amorphae* and Its Role in Aiding Phytostabilization by *Robinia pseudoacacia* in Copper Contaminated Soil

Xiuli Hao,[†,‡] Pin Xie,[†] Yong-Guan Zhu,[‡] Safyih Taghavi,[§] Gehong Wei,*[,†] and Christopher Rensing*[,‡,∥]

[†]State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China

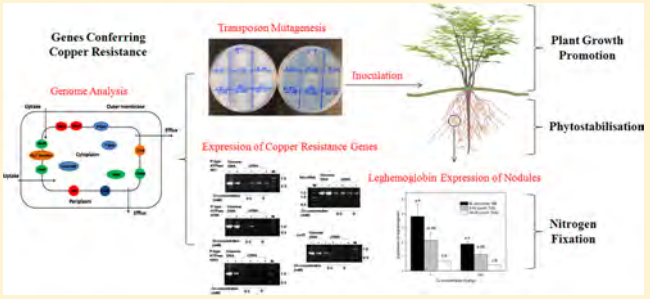
[‡]Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China

[§]FMC Agricultural Solutions, Research Triangle Park 27709, United States

[∥]Department of Plant and Environmental Science, University of Copenhagen, Frederiksberg DK-1871, Denmark

Supporting Information

ABSTRACT: The legume−rhizobium symbiosis has been proposed as an important system for phytoremediation of heavy metal contaminated soils due to its beneficial activity of symbiotic nitrogen fixation. However, little is known about metal resistant mechanism of rhizobia and the role of metal resistance determinants in phytoremediation. In this study, copper resistance mechanisms were investigated for a multiple metal resistant plant growth promoting rhizobium, *Mesorhizobium amorphae* 186. Three categories of determinants involved in copper resistance were identified through transposon mutagenesis, including genes encoding a P-type ATPase (CopA), hypothetical proteins, and other proteins (a GTP-binding protein and a ribosomal protein). Among these determinants, *copA* played the dominant role in copper homeostasis of *M. amorphae* 186. Mutagenesis of a hypothetical gene *lipA* in mutant M_{lipA} exhibited pleiotropic phenotypes including sensitivity to copper, blocked symbiotic capacity and inhibited growth. In addition, the expression of *cusB* encoding part of an RND-type efflux system was induced by copper. To explore the possible role of copper resistance mechanism in phytoremediation of copper contaminated soil, the symbiotic nodulation and nitrogen fixation abilities were compared using a wild-type strain, a *copA*-defective mutant, and a *lipA*-defective mutant. Results showed that a *copA* deletion did not affect the symbiotic capacity of rhizobia under uncontaminated condition, but the protective role of *copA* in symbiotic processes at high copper concentration is likely concentration-dependent. In contrast, inoculation of a *lipA*-defective strain led to significant decreases in the functional nodule numbers, total N content, plant biomass and leghemoglobin expression level of *Robinia pseudoacacia* even under conditions of uncontaminated soil. Moreover, plants inoculated with *lipA*-defective strain accumulated much less copper than both the wild-type strain and the *copA*-defective strain, suggesting an important role of a healthy symbiotic relationship between legume and rhizobia in phytostabilization.



INTRODUCTION

Copper contaminated soil from agricultural and industrial activities including mining, use of copper fungicides and fertilizer, as well as in sewage sludge has become an important environmental concern.[1,2] Although copper is essential for living organisms, exposure to excess copper leads to severe cell damage because of the generation of free radical species and the inactivation of enzymes often due to its property as a soft metal and thereby represents a great threat to the environment, microbes, and animal and human health.[3] To overcome copper toxicity to cells, bacteria have evolved an extensive network for copper transport and intracellular distribution. Evidenced by the facts that copper-requiring proteins are exclusively located within the cytoplasmic membrane or in the periplasmic space, cytoplasmic copper is generally unncessary and toxic for most bacteria.[4,5] Therefore, microbes strive to pump out cytoplasmic copper to protect cellular components and metabolic activities within the cytoplasm. The three previously described principle mechanisms that maintain intracellular copper homeostasis included efflux, sequestration, and Cu(I) oxidation.[6] In addition, induced global responses to copper stress including responses against oxidative stress and physiological and biochemical adaption have also been identified through transcriptomic and proteomic profilings.[7,8] In the model organism *Escherichia coli*, three essential chromosomal elements

Received: October 10, 2014
Revised: December 24, 2014
Accepted: January 16, 2015
Published: January 16, 2015

DOI: 10.1021/es504956a
Environ. Sci. Technol. 2015, 49, 2328−2340

responsible for detoxification of cytoplasmic and periplasmic copper resistance have been well characterized.[6,9] As a central component of copper homeostasis within the cytoplasm, the Cu(I)-transporting P-type ATPase CopA exports excessive Cu(I) from the cytoplasm to the periplasm.[10] CueO is a multicopper oxidase (MCO) that protects periplasmic enzymes from Cu(I)-mediated toxicity by oxidizing the highly toxic Cu(I) to the less toxic Cu(II) under aerobic conditions.[6] Both CopA and CueO are regulated by the transcriptional regulator CueR that belongs to the broadly distributed bacterial MerR family.[11] The multicomponent copper transporting system CusCFBA that is activited by the two-component regulatory system CusRS, is responsible for Cu(I) detoxification of the periplasm by exporting Cu(I) to the extracellular space.[12,13] In addition, plasmid borne *pco* determinants confer additional copper resistance in the periplasm where PcoA functions as CueO. The *pco* operon is regulated by the two-component system PcoRS sensing periplasmic Cu(I). Detailed descriptions of the function, structure, regulation, and interaction of Pco proteins have been discussed extensively in previous studies.[14,15] The protein families responsible for copper homeostasis and resistance are usually highly conserved, ubiquitous and abundant in bacterial genomes.[5] However, most of what is known about bacterial copper homeostasis is based on the work in the model organisms *Escherichia coli* and *Enterococcus hirae*, whereas what is known about the molecular mechanisms of copper tolerance of rhizobia is limited.[16,17]

Phytoremediation based on the use of plant−microbe consortia to extract or stabilize the contaminated environment has been proven to be a reliable and sustainable approach for the cleanup of heavy and transition metals and restoration of soil quality.[18,19] However, metal toxicity together with deficiency of phosphorus and/or nitrogen are two major factors limiting the growth of plants used in phytoremediation of contaminated soil, as exemplified by the barren, unvegetated mine tailings lasting for centuries. As pioneer plants, legumes have shown great potential to revegetate degraded soils in previous studies. There are several advantages of using legumes in phytoremediation, especially legume trees, including deeper roots system, extensive plant cover, and the ability to obtain nitrogen through a symbiotic relationship with N_2-fixing rhizobia.[20,21] Moreover, legume−rhizobium symbiosis lessens metal toxicity and renders plants more tolerant to excess metal, as reflected in the increase of plant biomass production, germination rate, elongation rate, antioxidant capacity, and metal translocation.[22,23] However, most rhizobia and legumes are quite sensitive to metals and therefore represent a "bottleneck" for successful recovery of such soils.[24,25] In this case, metal resistance determinants are crucial for rhizobia to survive and maintain a certain threshold for effective nodulation. Recent studies have isolated some metal resistant rhizobia that are appropriate to assist phytostabilization of mine tailings.[26,27] Both metal tolerant and metal sensitive rhizobia were screened and illustrated the importance of rhizobial metal resistance on effective symbiosis and phytoremediation.[28] However, because information about metal resistance mechanisms of rhizobia used in previous studies is still limited, it is not known which role rhizobial resistance to toxic metals plays for a given plant and symbiotic partner to survive in a metal contaminated environment. In addition, the enhanced phytoremediation activity of rhizobia may be due to either/both bacterial metal resistance or/and plant growth pormoting traits, but the roles of these two activities in plant survival and phytoremediation is still unclear.

We recently isolated and sequenced the genome of copper resistant *Mesorhizobium amorphae* 186 that significantly improved growth of *Robinia pseudoacacia* in copper contaminated soil.[29] Although genomic sequence data gave us an overview of genes encoding putative proteins involved in copper resistance in *Mesorhizobium*, functional evidence had not been provided. In this study, molecular determinants in *M. amorphae* responsible for copper resistance were investigated. Copper sensitive mutants generated by transposon mutagenesis were used to better understand the role of specific rhizobial copper resistance determinants on symbiosis and phytostabilization capability under copper stress.

MATERIALS AND METHODS

Bacterial Growth Conditions. *M. amorphae* strain 186 was grown in TY medium (5 g Tryptone, 3 g yeast extract, 0.46 g $CaCl_2$, and 15 g agar per liter) at 28 °C as previously described.[30] *E. coli* BW20767 (pRL27) was grown in Luria−Bertani (LB) medium with 50 μg/mL kanamycin (Km) at 37 °C for conjugation. *E. coli* EC1000 *pir*-116 was a recipient strain for electro-transformation. Metabolic properties including carbon and nitrogen utilization, resistance to antibiotics, heavy metals and salt, IAA production, siderophore secretion, and phosphorus solubilizing activity were determined according to methods reported previously.[31]

Tn5 Mutagenesis and Analysis of Copper Sensitivity. *M. amorphae* strain 186 was mutagenized by Tn5 from donor strain *E. coli* BW20767 with suicide plasmid pRL27 (Km^r).[31] Selective SM agar medium (0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2$, 0.5 g KNO_3, 0.5 g K_2HPO_4, 0.1 g NaCl, 10 g mannitol, 75 mg pantothenic acid, 75 mg biotin, 75 mg thiamine, and 15 g agar per liter) with 75 μg/mL Km was used for Tn5 insertion mutants screening after biparental mating. To obtain copper sensitive mutants, all insertion mutants from SM medium with 75 μg/mL Km were picked up onto TY plates supplemented with 0, 1.0, and 1.6 mM Cu^{2+}. Mutant clones showing weak growth or no-growth on 1.0 and 1.6 mM Cu^{2+} TY plates were recovered and further used to determine Tn5 insertion sites.

Whole genomic DNA of each copper sensitive mutant was isolated using the Blood & Cell Culture DNA Mini kit (Qiagen, MD, USA) and completely digested by HpyCH4IV (New England Biolabs, MA, USA). Digested DNA was heated at 65 °C for 20 min to inactivate HpyCH4IV. The fragments were ligated with approximately 600 pmol of a Y-shaped linker, which was prepared by annealing two oligonucleotides as reported before.[32] The ligation mixture was purified by QIAquick PCR Purification Kit (Qiagen, MD, USA) and used as template for PCR amplification. Gradient PCR was used to determine the optimal annealing temperature. Only the fragments containing the mini-Tn5 sequence could be amplified in the PCR reaction with specific Tn5 primer (tpnRL13−2:5′-CAGCAACACCTTCTTCACGA-3′) and Y linker primer (Py: 5′-GGATTTGCTGGTCGAATTCAAC-3′).

M. amorphae strain 186 and the copper sensitive mutants were grown to midexponential phase and cell suspensions were prepared for the same OD_{600} (optical density at 600 nm) values. The initial cell suspensions were inoculated into TY medium supplemented with different concentrations of Cu^{2+} and Ni^{2+} (1% inoculum). The cultures were incubated at 30 °C with agitating at 250 rpm until wild-type culture without metal

DOI: 10.1021/es504956a
Environ. Sci. Technol. 2015, 49, 2328−2340

Table 1. Primers Used for RT-PCR

gene	accession #	primer
M. amorphae		
cusB 1147	EHH12628	cusBA: 5′- CGGGAGCGGGCGGATACT - 3′
		cusBF: 5′- GCCTTCAGGTTGCTTTCG - 3′
MCO 1452	EHH12634	1452A: 5′- GTTGCTAGGTGCGGGAGC - 3′
		1452F: 5′- TGAGGTTGAGGGGTTTCG - 3′
MCO 1458	EHH12641	1458A: 5′- GATCATGGACTTCGACACG −3′
		1458F: 5′- CGCCCAAGTCGCTCAG - 3′
MCO 6349	EHH03674	6349A: 5′- TGGTTACAAGACGACAAT - 3′
		6349F: 5′- GGGACAACGGTCATTGCATC - 3′
copG 6352	EHH03677	copGA: 5′- ATCCGTGGTGCGGCTGCT - 3′
		copGF: 5′- CGCTTCCATCCTTGCTAA - 3′
16S rRNA		P_1: 5′- AGAGTTTGATCCTGGCTCAGAACGAACGCT - 3′
		P_6: 5′- TACGGCTACCTTGTTACGACTTCACCCC −3′
R. pseudoacacia		
leghemoglobin		yz441F: 5′- CTCAAGGCTCACGCTGAAAAGG - 3′
		yz441R: 5′- TTGCTCAATTCGTCGCTCCATT - 3′
18S rRNA		18sF: 5′- TAGTTGGTGGAGCGATTTGTC - 3′
		18sR: 5′- CAGAACATCTAAGGGCATCACAG - 3′

ions reached the stationary phase. OD_{600} of all samples were determined with a PerkinElmer UV/vis spectrophotometer.

Gene Expression Analyses of Cus and MCO Determinants. The draft genome sequence of *M. amorphae* strain 186 had been reported with the accession number AGSN00000000 in GenBank.[29] Expression of genes encoding putative copper resistance determinants in *M. amorphae* 186 genome were assessed by reverse transcriptase (RT) PCR. These genes included *cusB* encoding a component of a copper efflux system of the resistance-nodulation-cell division protein (RND) family, *copG* and three genes encoding multicopper oxidases (MCOs). Total RNA was isolated from midexponential phase cells treated with 0 or 0.5 mM Cu^{2+}, Zn^{2+}, and Mn^{2+} for 15 min respectively using the RNeasy Mini Kit (Qiagen, Maryland, USA). Procedures including DNA elimination, verification of DNA absence in total RNA, cDNA synthesis as well as the PCR program were performed as described previously.[31] Primers used to monitor transcription of genes were designed and listed in Table 1. 16S rRNA gene was used as a positive control. PCR amplifications were analyzed by 1% agarose gel electrophoresis.

Plant Growth, Macronutrient Content and Copper Accumulation. Seeds of *Robinia pseudoacacia* were surface sterilized and germinated using the procedures described previously.[31] Germinated seedlings were sown in pots filled with sterilized mixture of vermiculite and perlite (3:2, v/v) and then incubated in the greenhouse. When the first main leaf grew out, suspensions of either *M. amorphae* strain 186 or of two copper sensitive mutants, M_{copA}-1 and M_{lipA}, were added to each plant root with a final concentration of 10^8 CFU per root. Plants were subjected to 0, 100, or 200 mg/kg copper treatments with at least three replicates. Quantified Fahraeus nitrogen-free nutrient solution was used to water plant each week. After the plants were harvested after 45 days, the number of nodules was counted and dry weight of shoots and roots were measured. Nodule samples of different treatments were quickly frozen in liquid N_2 after harvest and then stored at −80 °C for further tests.

Dried shoots and roots were ground, homogenized, and then separated into two equal parts for determination of copper and total nitrogen content, respectively. Briefly, dried plant tissues were burned to dry ash in a muffle furnace at 600 °C for 2−3 h. Dry ash was then dissolved in 50% HNO_3 and diluted to the required analytical range for copper determination. Copper concentration was analyzed with atomic absorption spectrophotometry (AAS, Z-5000, Hitachi, Tokyo, Japan) using an external standard method. 0.1 g of pure copper was dissolved by 50 mL of 50% HNO_3 and diluted to 100 μg/mL copper standard solution. Reference material bush branches and leaves (GBW-07602, China) were used for copper extraction quality control. For N content determination, powdered shoot and root samples were digested with concentrated H_2SO_4−H_2O_2 using the method described by Horneck and Miller.[33] Total nitrogen in roots and shoots were determined using a continuous flow analytical system (3-AA3 AutoAnalyzer, Bran +luebbe, Germany).

Nodule Structure and Leghemoglobin Expression. Fresh nodules from *R. pseudoacacia* inoculated with *M. amorphae* 186 and two mutants in the presence of 0, 100, and 200 mg/kg copper were fixed in FAA solution (90 mL 70% ethanol, 5 mL acetic acid, and 5 mL methanol) for 12 h before staining by Eosin Y. Dehydrating and clearing processing were carried out through a graded ethanol series and chloroform series, respectively, followed by embedding and sectioning of paraffin blocks. Nodule sections of 5−10 μm thickness were then deparaffinated, rehydrated and stained by 0.5% (w/v) toluidine blue solution for final observation with a CX31RTSF biological microscope (Olympus, Japan).

Total RNA was isolated from frozen nodule samples of each treatment using TRIzol Reagent (Takara, Dalian, China). cDNA was prepared using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. Quantitative PCR was performed in triplicate using SYBR Premix Ex *Taq*II (Tli RNaseH Plus) kit (Takara, Dalian, China) in a CFX96 Real-Time PCR Detection System (BIO-RAD, USA). The thermocycling condition were 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. 18S rRNA was used as housekeeping genes for data normalization. The specific primer pairs for leghemoglobin amplification are listed in Table 1.

Data Analysis. Statistical analyses were performed with SPSS 18.0 software package (Chicago, USA). A one-way analysis of variance (ANOVA) was used to assess differences

DOI: 10.1021/es504956a
Environ. Sci. Technol. 2015, 49, 2328−2340

among individual inoculation treatments on plant growth and copper accumulation under one copper concentration treatment. Effects of inoculation combined with copper stress on plant copper accumulation were analyzed using a two-way ANOVA. Duncan's test ($p < 0.05$) was carried out for multiple comparisons. All data presented in this study were the means ± standard deviation (SD) of three independent replicates.

RESULTS

Genes Involved in Copper Homeostasis in *M. amorphae* Were Identified by Transposon Mutagenesis. After Tn5 insertion, eight copper sensitive mutants of *M. amorphae* were individually screened on TY agar medium supplemented with 1.0 and 1.6 mM Cu^{2+}. Among the eight mutants, five mutants (M_{copA}-1, M_{copA}-2, M_{copA}-3, M_{HP}-1, and M_{lipA}) showed the greatest sensitivity to 0.8 mM Cu^{2+}, while the tolerance of mutants M_{GTP} and M_{RP} declined gradually with increasing Cu^{2+} concentrations without a sharp turning point when compared to other mutants (Figure 1A). To identify whether the observed metal sensitivity is specific for Cu^{2+}, sensitivity to Ni^{2+} was also tested. Except for mutant M_{copA}-2, 0.6 mM Ni^{2+} inhibited the growth of most mutants in different degrees when compared to the wild-type strain. Mutants M_{HP}-1 and M_{RP} were sensitive even to 0.4 mM Ni^{2+}, indicating these mutations were not exclusively affecting resistance to Cu^{2+} (Figure 1B). Moreover, a stimulated growth was found in all mutants and wild-type strain at the range of 0−0.2 mM Cu^{2+} and Ni^{2+}.

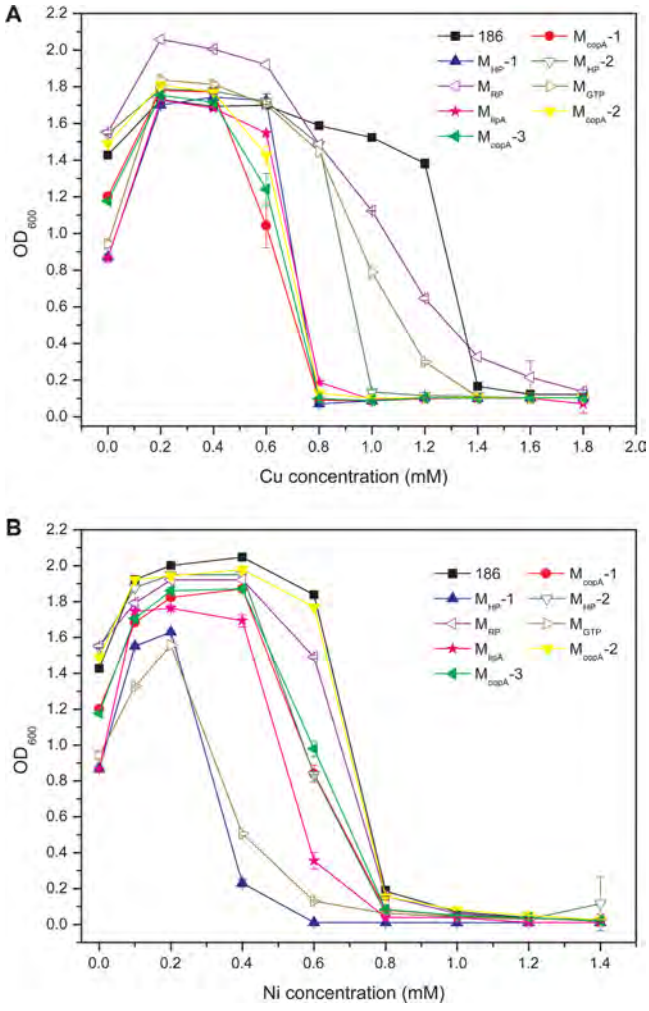


Figure 1. Response of *M. amorphae* wild-type strain 186 and Tn5 insertional mutants to Cu^{2+} and Ni^{2+} stress. Cells were grown in TY medium supplemented with increasing concentrations of Cu^{2+} (A) and Ni^{2+} (B) at 30 °C. Samples were taken until the culture of the wild-type strain without metal stress reached stationary phase. Data are the mean ± SD (n = 3).

Tn5 insertion sites located on the whole genome of *M. amorphae* 186 could be sorted into three categories, one being a specific P-type ATPase (Accession number: EHH02252, mutants M_{copA}-1, M_{copA}-2, and M_{copA}-3), another one defined more loosely under hypothetical proteins (Accession number: EHH11276, EHH13351, and EHH13838 for mutants M_{HP}-1, M_{HP}-2, and M_{lipA}) and finally one insertion encoding a GTP-binding protein (Accession number: EHH11454, mutant M_{GTP}) and a ribosomal protein (Accession number: EHH11235, mutant M_{RP}) (Figure S1, Supporting Information). For the gene encoding a P-type ATPase, subsequently named as *copA*-6910, Tn5 inserted into the same gene at different locations in three mutants, leading to a slight difference in growth under copper stress among these mutants (Figure 1A). Compared to insertions near the middle and the end of the gene (mutants M_{copA}-1 and M_{copA}-3), insertion at the beginning of the gene encoding the N-terminus of CopA-6910 (mutant M_{copA}-2) did not completely eliminate copper resistance mediated by CopA-6910. In agreement with CopA in other bacteria (Figure 2), CopA-6910 in *M. amorphae* contains three conserved domains including phosphatase domain (TGE), phosphorylation domain (P-domain, DKTGT), and the ATP-binding domain (N-domain, GDGIN)[10,34] Eight transmembrane helixes (TMHs) were predicted in CopA-6910, including one typical Cu(I) binding motif CPCALG in TMH 6. Phosphatase domain connects TMH4 and TMH5, while P domain and N-domain connect TMH6 and TMH7. Besides CPC in TMH6, CopA-6910 also contains other conserved amino acids including NY in TMH7 and MXSS in TMH8. These conserved sequences were reported to be responsible for selectively binding of Cu(I)/Ag(I).[35] In general, CXXC N-MBDs and histidine-rich N-MBDs are two types of N-terminal metal binding domains (N-MBDs) reported in Cu (I) transporting P_{1B}-type ATPase.[36,37] As shown in Figure 2, *E. coli* CopA has two N-terminal CXXC motifs ($GLSC_{14}GHC_{17}$ and $GMSC_{110}ASC_{113}$). However, these two MBDs are not essential for either copper resistance or transport.[38] The lack of a CXXC motif was found in *Legionella pneumophila* CopA.[39] Here, only one N-terminal CXXC motif was found in CopA-6910.

Three individual genes encoding hypothetical proteins with unknown function were interrupted in mutants M_{HP}-1, M_{HP}-2, and M_{lipA}, respectively. As shown in Figure S1 (Supporting Information), genes encoding the cytochrome c maturation (Ccm) complex were adjacent to a hypothetical gene (*lipA*, Accession number: EHH13838) interrupted in mutant M_{lipA}. Interruption of *lipA* resulted in a high sensitivity to copper, which was comparable to *copA-6910*-mediated loss of copper resistance in *M. amorphae* 186. Genes encoding a putative GTP-binding protein and a putative ribosomal protein were interrupted in mutant strains M_{GTP} and M_{RP}, respectively. In addition, a reduced growth rate in TY medium without copper supplement was also observed in mutants M_{HP}-1, M_{RP}, and M_{lipA}.

CusB Determinant Identified in the Draft Genome of *M. amorphae* Strain 186 Was Induced by Copper. Besides

DOI: 10.1021/es504956a
Environ. Sci. Technol. 2015, 49, 2328−2340

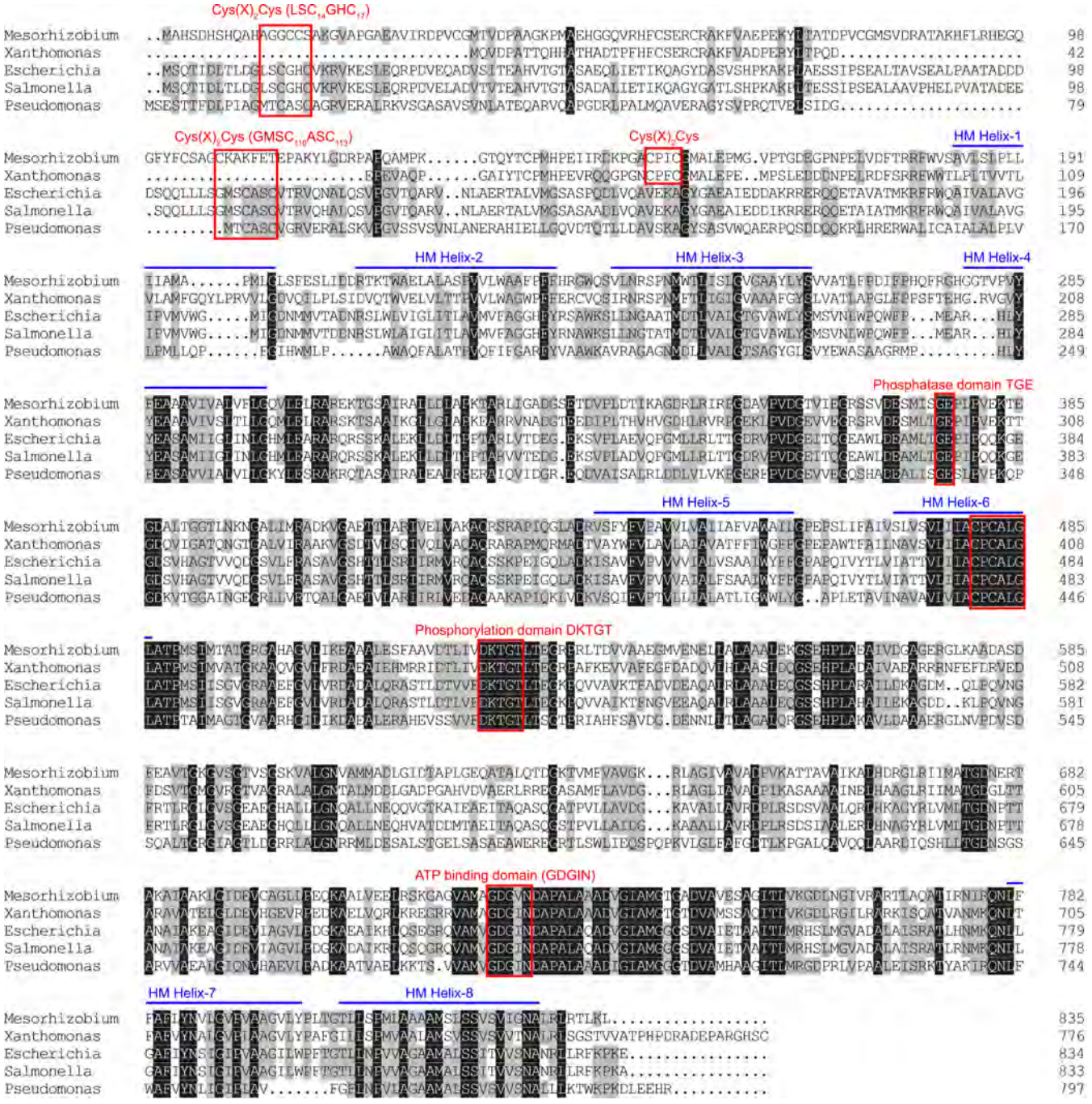


Figure 2. Amino acid sequences alignments of CopA-6910 in *M. amorphae* with CopA from other copper resistant bacteria. CopA sequences used for alignment were from *Mesorhizobium, Mesorhizobium amorphae* (Accession number: EHH02252, this study); *Xanthomonas, Xanthomonas vesicatoria* (Accession number (RefSeq): WP_005995730); *Escherichia, Escherichia coli* (Accession number (UniProt): Q59385);[10] *Salmonella, Salmonella enterica* (Accession number (UniProt): Q8ZR95);[73] *Pseudomonas, Pseudomonas putida* (Accession number: AAM88668).[74] The right numbers correspond to the positions of the last residue in the aligned sequences. Sequences in the boxes correspond to N-terminal CXXC motifs, the typical Cu(I) binding motif CPCALG in TM helix 6 and three conserved domains as labeled above the boxes.

the CopA-6910 identified by transposon mutagenesis, there were other putative copper resistance determinants predicted on the *M. amorphae* 186 genome, including CusAB a member of the RND family, one MCO operon containing genes encoding an outer membrane protein, a multicopper oxidase [MCO], a blue copper azurin-like protein [copper tolerance protein] and a CusF-type periplasmic copper chaperone, as well as another copy of the MCO operon together with genes encoding both CopG and a CusF-type periplasmic copper chaperone (Figure 3A).[29] To identify if these predicted determinants function in copper resistance, RT-PCR was conducted with genes encoding putative CusB, CopG, and three genes encoding MCOs (MCO1452, MCO1458, and MCO6349) using the wild-type strain induced by 0.5 mM Cu^{2+}, Zn^{2+}, and Mn^{2+} (Figure 3B). 16S rRNA gene was amplified as positive control. The expression of CusB (Accession number: EHH12628) was only up-regulated in wild-type strain when treated with 0.5 mM Cu^{2+}. No inducible

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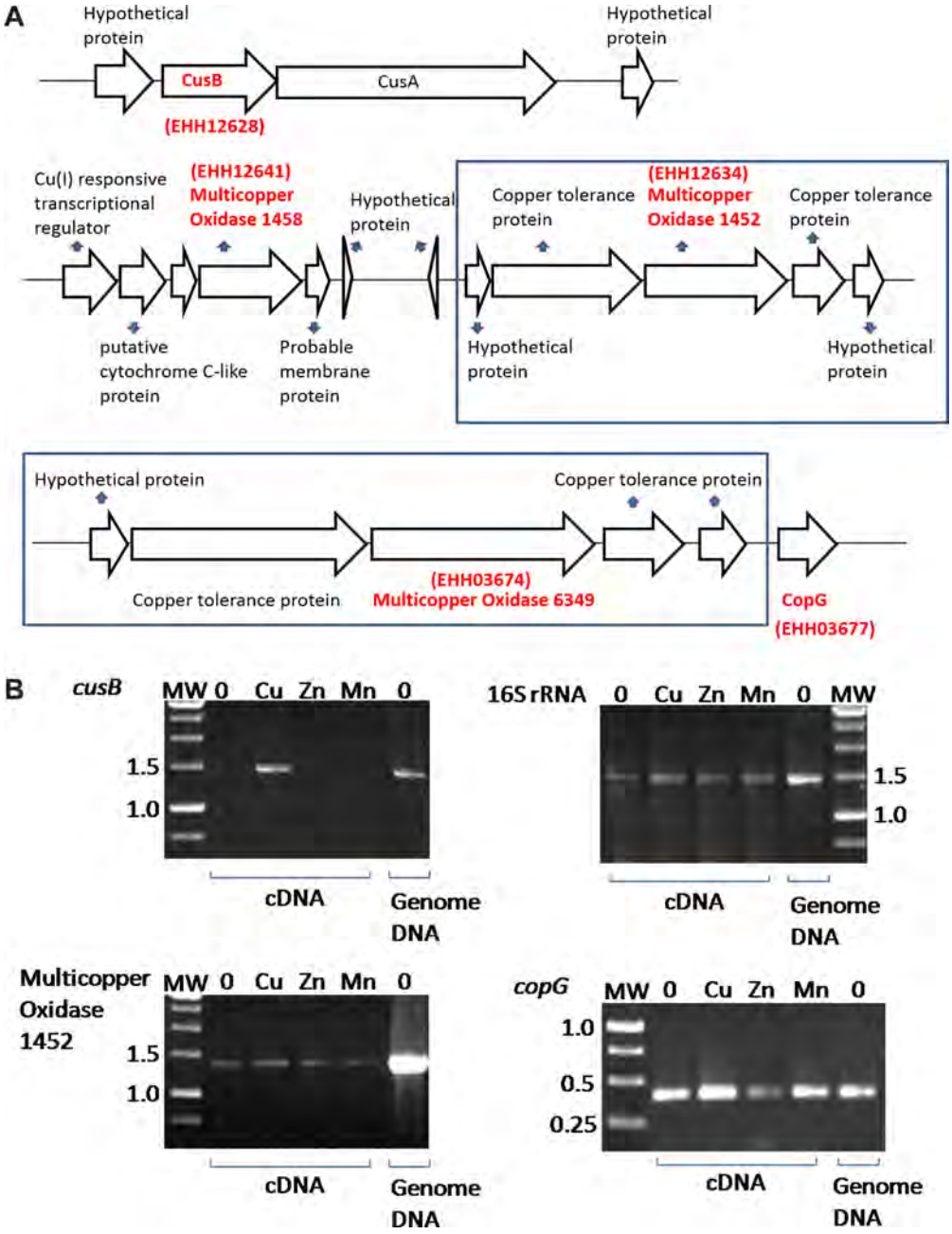

Figure 3. Expression of genes encoding predicted copper resistance determinants on the genome of *M. amorphae* 186. (A) Physical map of *cusBA* and MCOs operons. Operons in rectangle are two copies of MCOs. (B) Expression of *cusB*, multicopper oxidase encoding gene *MCO*, and *copG*. Genes encoding putative CusB, MCOs, and CopG were amplified using cDNA extracted from *M. amorphae* 186 induced by 0 and 0.5 mM Cu^{2+}, Zn^{2+}, and Mn^{2+} for 15 min. 16S rRNA was amplified as a control. MW was molecular size standards in kbp.

expression was found in Zn^{2+} or Mn^{2+} treatments, suggesting the function of CusB is exclusively in copper homeostasis. CopG (Accession number: EHH03677) and MCO1452 (Accession number: EHH12634) displayed constitutive expression under all metal treatments. However, amplifications of MCO1458 (Accession number: EHH12641) and MCO6349 (Accession number: EHH03674) failed despite treatment by 0.5 or 1.0 mM Cu^{2+}, Zn^{2+}, or Mn^{2+} with 5, 15, or 90 min (data not shown).

Mutagenesis of *lipA* Affected Symbiotic Relationship with *R. pseudoacacia*. To determine effects of *copA*-6910 and *lipA* on the symbiotic capacity of *M. amorphae* 186, *R. pseudoacacia* seedlings were inoculated with wild-type strain 186, mutant M_{copA}-1 (*copA*::Tn5), and mutant M_{lipA} (*lipA*::Tn5). Plant weight, nodule numbers, total N content, and leghemoglobin expression were determined to evaluate the symbiotic efficiency. *M. amorphae* wild-type strain 186 and the two mutants were able to form a symbiosis with *R. pseudoacacia* without metal stress. *M. amorphae* 186 was able to produce well-defined rod-shaped pink nodules. Most of the pink nodules grew separately on the first two lateral roots in the upper zone (Figure 4A). No significant difference was observed in nodule production between wild-type strain and mutant M_{copA}-1 (*copA*::Tn5) (Table 2). However, the number of pink nodules produced by mutant M_{lipA} (*lipA*::Tn5) decreased significantly ($P < 0.05$) compared to nodule numbers generated

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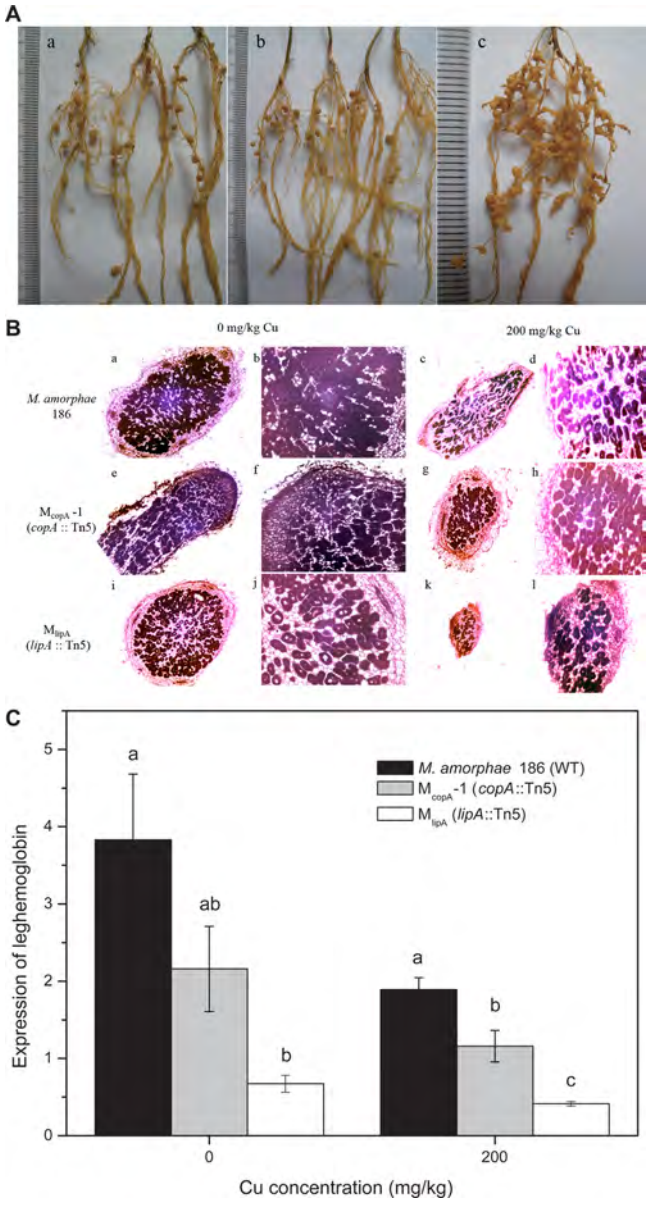

Figure 4. Symbiosis of *R. pseudoacacia* with *M. amorphae* wild-type strain, *copA*-insertional and *lipA*-insertional mutant. (A) Effects of *copA* or *lipA*-insertion on nodulation. Nodules of *Robinia* plants inoculated with *M. amorphae* wild-type strain 186 (a), mutant M_{copA}-1 (*copA*::Tn5) (b), and M_{lipA} (*lipA*::Tn5) (c) at 40 DPI (days post inoculation). (B) Light micrographs of nodule sections produced by *M. amorphae* wild-type strain 186 (a–d), mutant M_{copA}-1 (*copA*::Tn5) (e–h), and M_{lipA} (*lipA*::Tn5) (i–l) with 0 and 200 mg/kg copper. (C) Leghemoglobin expression of nodules inoculated with *M. amorphae* 186, mutant M_{copA}-1 (*copA*::Tn5) and M_{lipA} (*lipA*::Tn5) with 0 and 200 mg/kg copper in soil. Three independent biological samples containing 20–25 nodules per sample were used for RNA extraction. Data are the mean ± SD (n = 3).

by wild-type strain and mutant M_{copA}-1 (*copA*::Tn5). Moreover, most nodules generated by M_{lipA} (*lipA*::Tn5) were small, round, and distributed throughout the root system in clusters. The histological organization of nodules showed that the infected cells in nodule tissue induced by M_{lipA} (*lipA*::Tn5) were much less than infected cells in nodules produced by the wild-type strain (Figure 4B). Starch grains could be found accumulated in uninfected cells of M_{lipA} (*lipA*::Tn5) induced nodule tissue. The level of leghemoglobin expression in nodules is one of the important indicators reflecting nitrogen fixation ability of rhizobia. As shown in Figure 4C, leghemoglobin expression in nodules produced by mutant M_{copA}-1 was reduced but the difference was not significant when compared to nodules produced by wild-type strain without copper stress. However, *lipA* interruption in mutant M_{lipA} impacted the expression of leghemoglobin, which correlated with the reduced number of pink nodules. It is notable that a high concentration of copper had a great inhibitory effect on the expression of leghemoglobin in nodules produced by all strains. Therefore, copper toxicity to plant leghemoglobin production was independent of inoculated rhizobia.

Because N-free nutrient solution was applied during the whole test, plant nitrogen content was used to reflect the nitrogen fixation ability of rhizobia. In all cases, although plant nitrogen contents were decreased with the increase of copper concentration in the medium, nitrogen contents in both shoots and roots were nearly doubled in plants inoculated with the wild-type strain 186 and mutant M_{copA}-1 (*copA*::Tn5) compared to the control without inoculation (Figure 5), indicating a similar nitrogen fixation ability of the two strains. These increases were also reflected in about 76.31% and 38.40% higher shoot and root dry weight of plants with both wild-type strain and mutant M_{copA}-1 in uncontaminated soil (Figure 5). However, in the absence of copper, only a 38.75% increase of nitrogen content in shoots was observed in plants inoculated with mutant M_{lipA} (*lipA*::Tn5). The increased nitrogen content in shoots of plants inoculated with mutant M_{lipA} was far less than in shoots of plants inoculated with the wild-type strain or mutant M_{copA}-1 (nearly 100%). Meanwhile, inoculation of M_{lipA} increased the dry weight of shoots by 32.7% as compared to the control without inoculation. Presence of copper reduced the difference in the improvement of shoot nitrogen content stimulated by mutant M_{lipA} and wild-type strain. However, a significant difference was found between nitrogen content of roots (with nodules) inoculated with mutant M_{lipA} and wild-type strain ($P < 0.05$), which might be due to nitrogen reserves in nodules.

In conclusion, the improvement of plant growth by mutant M_{lipA} (*lipA*::Tn5) was much less than in the wild-type strain and mutant M_{copA}-1 (*copA*::Tn5) in the presence of 0 and 100 mg/kg copper. However, copper toxicity lessened the differences in plant growth promotion caused by rhizobia. Therefore, the interruption of *lipA* in mutant M_{lipA} not only affected

Table 2. Effect of *M. amorphae* Wild-Type Strain 186 and Mutants on Nodule Numbers with *R. pseudoacacia*[a]

	0 mg/kg Cu^{2+}		100 mg/kg Cu^{2+}		200 mg/kg Cu^{2+}	
strain	pink	white	pink	white	pink	white
186 (WT)	11 ± 2[a]	27 ± 7[a]	7 ± 2[a]	26 ± 8[a]	6 ± 1[a]	23 ± 7[a]
M_{copA}-1 (*copA*::Tn5)	8 ± 1[a]	22 ± 6[a]	4 ± 2[ab]	19 ± 8[a]	6 ± 1[a]	19 ± 6[a]
M_{lipA} (*lipA*::Tn5)	3 ± 1[b]	27 ± 7[a]	2 ± 0[b]	23 ± 3[a]	2 ± 0[b]	28 ± 2[a]

[a]Data are the mean ± SD ($n \geq 3$). Superscripts letters are significant differences ($P < 0.05$) between mutants and WT strain.

DOI: 10.1021/es504956a
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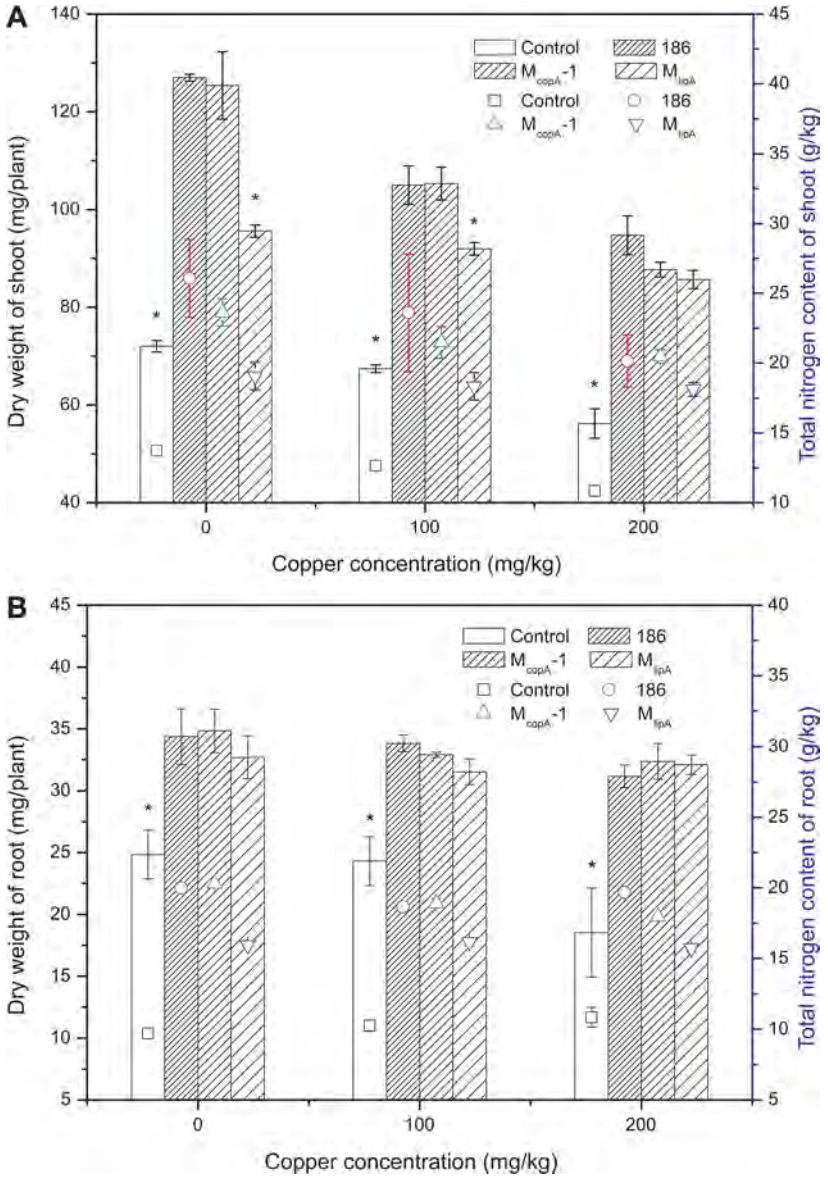

Figure 5. Effects of different inoculations on plant shoot growth (A) and root growth (B). *Robinia* plants inoculated with *M. amorphae* 186, mutant strains M_{copA}-1 (*copA*::Tn*5*), and M_{lipA} (*lipA*::Tn*5*) were grown in the presence of 0, 100, and 200 mg/kg copper. Bar graphs in each panel represent dry weights of shoot or root. The dot data points show nitrogen contents in plant shoot or root under different inoculation and copper treatments. Data are the mean ± SD ($n \geq 3$). An asterisk means significant difference ($P < 0.05$) observed from plant inoculated with mutant when compared to the wild-type strain (Duncan's test).

Table 3. Effect of *M. amorphae* Wild-Type Strain 186 and Mutants on Copper Accumulations in Shoots and Roots (With Nodules) of *R. pseudoacacia* Grown in Perlite−Vermiculite Supplemented with 0, 100, and 200 mg/kg Copper[a]

	shoot			root (with nodules)		
strain	0 mg/kg Cu	100 mg/kg Cu	200 mg/kg Cu	0 mg/kg Cu	100 mg/kg Cu	200 mg/kg Cu
186 (WT)	6.28 ± 1.05[a]	18.96 ± 2.58[a]	21.22 ± 2.33[a]	16.21 ± 0.93[a]	84.72 ± 2.55[a]	115.26 ± 12.46[a]
M_{copA}-1 (*copA*::Tn*5*)	5.43 ± 0.27[a]	19.20 ± 0.12[a]	20.61 ± 1.40[a]	18.23 ± 2.30[a]	71.90 ± 2.95[b]	104.16 ± 9.17[ab]
M_{lipA} (*lipA*::Tn*5*)	4.44 ± 1.27[a]	10.31 ± 0.80[b]	15.56 ± 1.84[b]	15.28 ± 2.27[a]	51.66 ± 10.55[c]	89.18 ± 4.50[b]
control[b]	6.69 ± 2.01[a]	9.08 ± 0.48[b]	15.01 ± 3.84[b]	16.14 ± 5.13[a]	35.18 ± 3.49[d]	61.77 ± 1.42[c]
analysis of variance						
Cu supply (Cu)	$P < 0.001$			$P < 0.001$		
strains (S)	$P < 0.001$			$P < 0.001$		
Cu × S	$P < 0.001$			$P < 0.001$		

[a]Values are the mean ± SD ($n \geq 3$). Superscripts letters are differences between different inoculations at the same copper treatment at the 0.05 level (Duncan). [b]Control plants without inoculation.

DOI: 10.1021/es504956a
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rhizobial copper resistance but also affected the ability of symbiotic nitrogen fixation in the *Robinia* plant.

Effects of *copA* and *lipA*-Defective Strains on Copper Distribution in Legume Tissues. To exclude other factors affecting plant growth, we monitored plant-growth-promoting traits including production of IAA, siderophore and acetoin production, ACC deaminase activity, as well as the mineral and organic phosphorus solubilization ability. No obvious differences in these plant-growth-promoting traits were found in both *M. amorphae* wild-type strain and its transposon mutants that were generated. Therefore, copper sensitive mutant M_{copA}-1 (*copA*::Tn5) and M_{lipA} (*lipA*::Tn5) generated by transposon mutagenesis were chosen to evaluate the role of copper resistance determinants and symbiosis in plant growth and copper removal capacity under different degrees of copper stress. Copper concentrations in both roots and shoots increased with the increase of external copper supplements. Rhizobia inoculation, no matter whether the wild-type strain or the mutants, promoted copper accumulation in *Robinia* but with a greater effect on roots than shoots (Table 3). Copper concentration in roots with wild-type strain 186 was more than 2 times higher than in the control pots without inoculation. Significant differences were observed in copper accumulation of roots with the wild-type strain and the two mutants in the presence of 100 mg/kg copper with the order 186 (WT) > M_{copA}-1 (*copA*::Tn5) > M_{lipA} (*lipA*::Tn5) > control (non-inoculation). However, when the copper concentration was increased to 200 mg/kg, there was no significant difference in copper accumulation of plant roots inoculated with mutant M_{copA}-1 and M_{lipA} ($P < 0.05$). In other words, the advantage of M_{copA}-1 (*copA*::Tn5) on copper accumulation promotion in the presence of 100 mg/kg copper was lessened to a similar level with M_{lipA} (*lipA*::Tn5) at high copper concentration. In addition, in the presence of 200 mg/kg copper, copper accumulations in roots inoculated with M_{lipA} were 22.63% and 14.38% less than copper accumulation in roots with the wild-type strain 186 and mutant M_{copA}-1, respectively, suggesting the role of symbiosis in phytostabilization was more critical than *copA*-mediated rhizobial copper resistance.

DISCUSSION

M. amorphae strain 186 is a multimetal resistant bacterium isolated from nodules of *Robinia pseudoacacia*. The genome of this rhizobium has been sequenced and analyzed in a previous study.[29] Here, we characterize mechanisms of copper resistance and the potential of harnessing these mechanisms for phytostabilization.

According to the analysis of mutants generated by transposon mutagenesis (Figure 1A), mutant M_{copA}-1 with P_{1B}-type ATPase CopA-6910-insertion (Accession number: EHH02252) was most sensitive to copper compared to other mutants in different genes, showing the critical role of this P_{1B}-type ATPase in copper resistance in *M. amorphae* 186. Similar to CopA in *E. coli*, CopA-6910 in *M. amorphae* contains three conserved domains (phosphorylation domain, amino-terminal domain and the nucleotide-binding domain) and one typical copper binding motif (CPCALG).[34] Amino acid alignment of CopA-6910 in *M. amorphae* showed 73.90% identity to ActP protein in the highly metal resistant *Mesorhizobium metallidurans* strain STM 2683 (Accession number: CCJ37800) isolated from a Pb/Zn mine[40] and 42.80% identity to CopA-like protein in copper resistant *Sinorhizobium meliloti* CCNWSX0020 (Accession number: EHK78920).[41] RT-PCR showed *cusB* (Accession number: EHH12628) was only induced by Cu^{2+} in *M. amorphae*, indicating that CusB was involved in maintenance of copper homoestasis (Figure 3B). In addition, hypothetical proteins with unknown functions disrupted in mutants M_{lipA} and M_{HP}-1 also showed a significant effect on bacterial copper resistance. Copper homeostatic systems are multilayered and intricate.[6,10,42] In *M. amorphae* 186, CopA-6910 is predicted to carry out Cu^{+} efflux from the cytoplasm to the periplasm, while CusAB is responsible for Cu^{+} detoxification of the periplasm by export to extracellular space. However, CopA-6910 and CusB constituted the main but certainly not the only copper detoxification system in *M. amorphae*. Interestingly, mutant M_{HP}-1 defective in hypothetical protein and mutant M_{RP} defective in ribosomal protein also showed great sensitivity to Ni^{2+} for unknown reasons.

The crucial roles of cbb_3-type cytochrome c oxidase (cbb_3-Cox) on bacteroid formation, nodulation, and nitrogen fixation during the rhizobium−legume symbiosis have been extensively studied.[43,44] During the process of cytochrome c maturation, the Ccm complex is responsible for the covalent attachment of heme to apocytochromes in the periplasm.[45] *ccmIEFH* is a complex operon encoding subunits of a heme-lyase. The *ccmE* gene encodes a heme chaperone shuttling heme between CcmC and CcmF; CcmH associates with CcmF and participates in keeping the apoprotein in a reduced state; CcmI functions as an assembly factor delivering apocytochrome to the heme ligation complex.[45,46] In some bacterial species such as *Rhizobium etli*[47] and *Rhodobacter capsulatus*,[48] *ccmF* could be transcribed independently from *ccmI* depending on different culture conditions. However, in other rhizobia such as *S. meliloti*,[49] *Rhizobium leguminosarum*,[50] and *Bradyrhizobium japonicum*,[51] the *ccmIEFH* genes are organized as an operon and mutations in these genes break down symbiotic nitrogen fixation completely due to the lack of symbiotic terminal cbb_3-Cox.[52] Interestingly, other then the cytochrome c maturation function, pleiotropic phenotypes of *ccm* mutants have been reported. These included copper sensitivity, reduced iron acquisition, lack of manganese oxidation, disturbed heme metabolism, impaired siderophore and IAA synthesis, as well as growth inhibition in rich media.[53,54] An early study[55] showed that CcmI and CcmF were required for copper resistance in *Pseudomonas fluorescens*. It was found that a single deletion of cytochrome was not fatal to bacteria due to alternative choices of respiratory oxidases, but the ability to grow in certain environments was impaired. Therefore, *ccm* genes were suggested to contribute to some cellular processes related to copper metabolism and thereby affected the management of copper toxicity indirectly. Recently, links between Ccm and cellular copper homeostasis have attracted increasing attention in recent years.[42,56,57] In this study, the *ccm* genes in *M. amorphae* 186 genome are arranged as three unlinked clusters *ccmABDG-ccmC-ccmIEFH*. A separate *ccmIEFH* operon from the *ccmABCDG* operon is common among bacteria within the α-family of proteobacteria.[54] The gene encoding a hypothetical protein that contains a conserved domain within the lipase LipA superfamily is located immediately upstream of *ccmI*. A previous study revealed that a consensus promoter sequence of *ccm* operon was found within the coding region of *lipA*.[47] Interruption of a *lipA*-like gene (*lipA*, Accession number: EHH13838) in the mutant M_{lipA} probably led to a polar effect on expression of downstream *ccm* genes in the *ccmIEFH* operon, and thereby resulted in slower bacterial growth in rich medium, a copper-sensitive phenotype of the bacteria, and

DOI: 10.1021/es504956a
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reduced symbiotic nitrogen fixation ability. As shown in Figure 1A, mutant M_{lipA} grew slowly in rich TY medium without metal but the growth rate was restored to the same level as the wild-type strain when the medium was supplemented with low concentration of Cu^{2+} or Ni^{2+}. However, mutant M_{lipA} could not survive when the copper concentration increased to 0.8 mM, indicating a high copper sensitivity. The reported copper sensitive phenotype cannot simply be explained by the loss of cytochrome c.[54,55] Two hypotheses underlying this phenotype can be formulated as (1) the deficiency of these genes led to a reduced level of heme and thereby affected enzymes related to oxidative stress resistance; (2) the presence of these genes might indirectly affect other processes required for copper homeostasis.[54,57] In addition, LipA is closely related to rhizobium−legume symbiosis. For example, the regulation of *lipA* transcription has been reported to affect nitrogenase activity through altering the TCA cycle in bacteroids.[58] Therefore, the impaired nitrogen fixation ability of mutant M_{lipA} could be due to interruption of *lipA* itself. However, questions as to whether the *ccmIEFH* in *M. amorphae* 186 works as a whole operon and deciphering the role of each *ccm* gene on rhizobial copper resistance still needs to be determined.

Nitrogen deficiency is one of the major limitations in plant productivity in metal contaminated soil.[28] However, the application of rhizobium−legume symbiosis systems for legume growth promotion and metal accumulation opens up a promising field of microbial assisted phytoremediation in metal contaminated soils. As the most important family of biological nitrogen fixing bacteria, rhizobia convert atmospheric nitrogen to plant utilizable ammonia by establishing a symbiosis with legumes, thereby improving nitrogen content and legume growth. Besides the function of nitrogen fixation, the symbiosis of plants with rhizobia led to more accumulated metal than in nitrogen fertilized plants.[23] Previous studies have shown the potential of different rhizobial species belonging to *Sinorhizobium*, *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium* for phytoremediation of metal contaminated soil.[41] Considering the survival advantage of metal tolerant bacteria in contaminated environments, most studies tended to concentrate on metal(loid)s tolerant rhizobia for phytostabilization of heavy metal contaminated soil.[21,27,28,59] Metal tolerance might protect rhizobia and thereby ensure the ability to build the effective symbiosis relationship under metal stress is maintained. Maynaud et al. found that the expression of *cadA* was induced by environmental zinc in both free-living and *Anthyllis vulneraria* nodules, supporting metal resistance determinants functioned well and were needed in the bacteroid under metal stress.[60] In addition, several attempts have been made to illustrate the importance of rhizobial metal resistance determinants on effective symbiosis, plant growth promotion and phytoremediation. Zribi et al. found that root biomass of *Medicago sativa* inoculated with zinc tolerant rhizobia was higher than plant with zinc sensitive rhizobia.[23] Compared to copper sensitive mutants, copper resistant wild-type *S. meliloti* CCNWSX0020 was more capable of assisting plant growth and phytostabilization.[41] However, the metal resistance mechanisms of rhizobia used in most studies mentioned above were not characterized and were thus unknown. Moreover, there were always other additional factors impacting plant growth or metal accumulation under metal stress in these studies, such as impaired IAA production. Therefore, our knowledge on how rhizobial metal resistance assists plant survival is still limited at present. In this study, copper resistance systems in *M. amorphae* were identified first. Besides symbiotic nitrogen fixation, no obvious differences in these plant-growth-promoting traits were found in *M. amorphae* wild-type strain, mutant M_{copA}-1 (*copA*::Tn*5*), and M_{lipA} (*lipA*::Tn*5*).

We found that excess copper indeed reduced the number of functional nodules and the infected cells in the nodules, which was in agreement with the responses of other reported rhizobia−legume symbioses to metal stress such as white lupin, soybean, and vigna.[25] The *copA* insertional mutant did not significantly impact the symbiotic nodulation capacity of *M. amorphae* with *R. pseudoacacia* in the uncontaminated or only low concentrations of copper containing soil (Table 2 and Figure 4B). Meanwhile, there was no prominent difference in nitrogen content of plant inoculated with the wild-type or the *copA*-deficient strain, suggesting similar nitrogen fixation abilities. However, the nitrogen content in roots with nodules inhabited by *copA*-deficient strain M_{copA}-1 decreased significantly at 200 mg/kg copper in soil. This result indicated that *copA* conferred copper tolerance to symbiotic bacteria which in turn partly benefited the nitrogen fixating performance at high levels of copper concentrations in soil and in nodules. In another ongoing analysis, we found that *chrA* in *M. amorphae* was essential for nodule formation under moderate chromate concentration but did not prevent the establishment of nodules at high chromate concentration (unpublished data). It is likely that the role of *copA* in symbiotic rhizobial protection was copper concentration-dependent. Further study is needed to investigate the applicable soil copper concentration in which *copA* is helpful for efficient nodulation.

Symbiotic nitrogen fixation was shown to be the key factor helping plant grow even in a copper contaminated environment. In rhizobia, cbb_3-Cox supports microaerobic respiration in endosymbiotic bacteroids.[61] During the process of symbiosis establishment and nitrogen fixation, two bursts of rhizobial division supported by ATP generation from the electron transport system were thought to be critical.[44] In this study, mutant M_{lipA} (*lipA*::Tn*5*) formed a symbiosis with *R. pseudoacacia*, but functional nodule numbers, infection cells, leghemoglobin expression and N fixation were reduced with or without copper stress. Mutagenesis of a hypothetical gene (*lipA*) which is likely linked to cbb_3-Cox not only affected copper resistance, but also affected the symbiotic relationship with *R. pseudoacacia*. This result was in accordance with previous reports that the loss of cytochrome c in rhizobia blocked nodule development and symbiotic nitrogen fixation.[62] The significantly reduced leghemoglobin expression in nodules produced by M_{lipA} (*lipA*::Tn*5*) might be due to heme biosynthesis deficiency.[63] Notably, leghemoglobin production was quite sensitive to copper stress in nodules generated by both the wild-type and two mutant strains (Figure 4C). Leghemoglobin has an excellent oxygen buffer capacity, allowing sufficient oxygen flux to bacteroids but simultaneously without inactivating oxygen-sensitive nitrogenase.[52,64] As a strong soft metal, copper produces the highly toxic hydroxyl radical through a Fenton-like reaction ($Cu^+ + H_2O_2 \rightarrow HO^- + HO^\bullet + Cu^{2+}$, $O_2^{\bullet-} + Cu^{2+} \rightarrow Cu^+ + O_2$).[5,65] Previous findings suggested that reduced nitrogen fixation caused by metal stress could be directly attributed to inhibition of nitrogenase function by inducing ROS production and affecting leghemoglobin/oxygen availability.[66,67] In addition, iron is an important cofactor required by many proteins essential for symbiotic nodulation and the nitrogen fixation process, such as

DOI: 10.1021/es504956a
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ferredoxin, nitrogenase, and leghemoglobin.[68,69] Excess copper disturbs iron−sulfur clusters and iron homeostasis, thereby negatively affecting various metabolic reactions including free radical detoxification, electron transfer, energy transduction, leghemoglobin function, and nitrogenase activity.[70]

Increasing plant biomass is one of the beneficial roles of plant growth promoting bacteria in plant responses to metal stress, which leads to a higher metal accumulation and diluted toxicity in plant.[71] In this study, *R. pseudoacacia* inoculated with *M. amorphae* 186 displayed both an increased biomass and copper accumulation compared to plants without inoculation in a copper-contaminated environment. A significantly declined shoot biomass was consistent with the impaired nitrogen fixation in plants with *lipA*-deletion mutant M_{lipA}. However, in the case of mutant M_{copA}-1 (*copA*::Tn5), there was no statistically significant difference in biomass between mutant and wild-type inoculated plants. A similar result was also reported in lentil plants inoculated with *Rhizobium leguminosarum dmeRF*-deficient mutant when exposed to Ni or Co excess. The *R. leguminosarum dmeF* gene encoding a divalent metal efflux facilitator is located downstream of the *dmeR* gene that encodes a transcriptional regulator.[17] A higher copper accumulation in *Robinia* roots than shoots was consistent with other reported symbiosis such as *Sinorhizobium*−*Medicago* and *Bradyrhizobium*−*Lupinus* in previous studies, suggesting a low translocation of copper from root to shoot.[25,41,72] Although a rhizobial *copA*-insertion in *M. amorphae* did not seriously affect its symbiotic plant biomass under copper stress, copper accumulation in roots with nodules generated by mutant M_{copA}-1 (*copA*::Tn5) reduced significantly compared to root inoculated with wild-type strain. However, the most dramatic decrease of copper accumulation was observed in roots with nodules generated by mutant M_{lipA} (*lipA*::Tn5). These results gave us a clear illustration that rhizobial copper tolerance determinants rendered its host plant a greater copper accumulation ability in copper contaminated soil. However, the capacity to form a healthy symbiotic relationship was far more important than copper resistance determinants of rhizobia in phytostabilization applications.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: physical map of transposon Tn5 interrupted genes related to copper resistance of *M. amorphae* 186. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Christopher Rensing. Tel: +45 29 356765. E-mail: chres@life.ku.dk.

*Gehong Wei. Tel: +86 029 87091175. E-mail: weigehong@nwsuaf.edu.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by projects from the 863 project of China (2012AA100402), the Cheung Kong Scholars Programme, the National Science Foundation of China (31125007 and 31370142) and China Postdoctoral Science Foundation (2014M551845). Sequencing was performed at the University of Arizona Genetics Core.

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DOI: 10.1021/es504956a
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