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Genome Sequence and Mutational Analysis of Plant-Growth-Promoting Bacterium *Agrobacterium tumefaciens* CCNWGS0286 Isolated from a Zinc-Lead Mine Tailing

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The plant-growth-promoting bacterium *Agrobacterium tumefaciens* CCNWGS0286, isolated from the nodules of *Robinia pseudoacacia* growing in zinc-lead mine tailings, both displayed high metal resistance and enhanced the growth of *Robinia* plants in a metal-contaminated environment. Our goal was to determine whether bacterial metal resistance or the capacity to produce phytohormones had a larger impact on the growth of host plants under zinc stress. Eight zinc-sensitive mutants and one zinc-sensitive mutant with reduced indole-3-acetic acid (IAA) production were obtained by transposon mutagenesis. Analysis of the genome sequence and of transcription via reverse transcriptase PCR (RT-PCR) combined with transposon gene disruptions revealed that ZntA-4200 and the transcriptional regulator ZntR1 played important roles in the zinc homeostasis of *A. tumefaciens* CCNWGS0286. In addition, interruption of a putative oligoketide cyclase/lipid transport protein reduced IAA synthesis and also showed reduced zinc and cadmium resistance but had no influence on copper resistance. In greenhouse studies, *R. pseudoacacia* inoculated with *A. tumefaciens* CCNWGS0286 displayed a significant increase in biomass production over that without inoculation, even in a zinc-contaminated environment. Interestingly, the differences in plant biomass improvement among *A. tumefaciens* CCNWGS0286, *A. tumefaciens* C58, and zinc-sensitive mutants 12-2 (*zntA*::Tn*5*) and 15-6 (low IAA production) revealed that phytohormones, rather than genes encoding zinc resistance determinants, were the dominant factor in enhancing plant growth in contaminated soil.

Plant-growth-promoting bacteria (PGPB) play a key role in host plant adaptation to metal-contaminated environments through triggering physiological changes in plant cell metabolism so that growing plants can tolerate high concentrations of transition or heavy metals (7, 26). PGPB have been isolated from various plants in heavy-metal-contaminated environments (3, 47–49). They were able to enhance plant growth, make plants more tolerant to heavy and transition metals, and thus accelerate phytoremediation by mechanisms including nitrogen fixation, nitrogen and phosphorus metabolism, and the production of siderophores, organic acids, 1-aminocyclopropane-1-carboxylate deaminase (ACC), and phytohormones such as indole-3-acetic acid (IAA), cytokinins, acetoin, and 2,3-butanediol (21, 41, 54). It was reported previously that the existence of high concentrations of metals selected for more metal resistant PGPB to resist these adverse environmental conditions (20, 26). However, it is often not known which trait of the PGPB, e.g., tolerance to heavy and transition metals, phytohormones, or a combination of the two, plays the dominant role in the survival of a given plant in a metal-contaminated environment.

Agrobacterium tumefaciens is a soilborne alphaproteobacterium belonging to the family *Rhizobiaceae* whose ability to induce crown gall tumors on dicotyledonous plants makes it of great concern worldwide. Recently, many nonpathogenic *A. tumefaciens* strains with plant-growth-promoting capacity have been isolated from the root nodules of various legumes (8, 50). Further studies revealed that as endophytic bacteria, *Agrobacterium* strains could also coexist with rhizobia in the nodules (30, 50). However, the role that endophytic *Agrobacterium* strains might play in the nodule, and whether they could serve as PGPB in phytoremediation, is still largely unknown. In this study, we isolated the metal-resistant and plant-growth-promoting *A. tumefaciens* strain CCNWGS0286 from root nodules of *Robinia pseudoacacia* collected from zinc-lead mine tailings in Gansu Province, China. Genes responsible for zinc resistance/homeostasis were first identified tentatively from the draft genome and were then further characterized through transposon mutagenesis combined with transcription analysis via reverse transcriptase PCR (RT-PCR).

To determine which trait (zinc resistance or IAA production capacity) played a more important role on assisting plant growth under zinc stress, two zinc-sensitive mutants with different IAA synthesis capacities were selected for plant-growth-promoting tests with the wild-type strain and with the reference strain *A. tumefaciens* C58 as a control. Furthermore, prediction of genes and potential pathways involved in plant growth promotion, combined with examination of metabolic properties, provided a better understanding of how *A. tumefaciens* strain CCNWGS0286 was able to assume a role as a beneficial endophytic bacterium for plant growth.

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MATERIALS AND METHODS

Isolation and identification of *Agrobacterium tumefaciens* CCNWGS0286. Bacteria were isolated from the nodules of *R. pseudoacacia*, which grew on a lead-zinc mine tailing in Gansu Province in northwestern China, by a standard method as described by Vincent (46). The resistant isolates were obtained by screening in yeast-mannitol agar (YMA) medium supplemented with different concentrations of $CuSO_4 \cdot 5H_2O$ or $ZnSO_4 \cdot 7H_2O$ at 28°C. MICs were determined using TY agar medium (5 g tryptone, 3 g yeast extract, 0.46 g $CaCl_2$, and 20 g agar per liter) amended with different concentrations of metals and incubated at 28°C for 3 days.

For identification, the 16S rRNA gene was amplified from the genomic DNA with primers P_1 (5′-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3′) and P_6 (5′-TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3′) as described by Tan et al. (43). *nodA* and *nifH* gene sequences were also analyzed as described by Tan et al. (43) and by Chen et al. (5) to see if the strain contained genes inducing the formation of root nodules. 16S rRNA gene sequences of type strains used for phylogenetic tree construction were aligned using the CLUSTAL W program. The phylogenetic tree was constructed using MEGA, version 3.0, by the neighbor-joining method with the Kimura 2 parameter model. The reliability of the branches was estimated by bootstrap analysis with 1,000 bootstrap replications.

Determination of metabolic properties. Strain CCNWGS0286 was tested for carbon and nitrogen utilization in WHITE medium (15) supplemented with different carbon and nitrogen sources (with $NaNO_3$ omitted, and with 10 g/liter mannitol as a carbon source) at a 0.1% (wt/vol) final concentration. Strains grown in YMA medium and WHITE medium without any carbon or nitrogen sources were used as positive and negative controls, respectively. WHITE medium without a carbon or nitrogen source (with $NaNO_3$ omitted) could be used for autotrophy and nitrogen fixation tests. Other resistance tests, such as antibiotic and salt tests, were carried out on YMA medium with different concentrations of antibiotics and NaCl, respectively. IAA production tests proceeded for 7 days at 28°C with shaking at 200 rpm as described previously (16), and IAA concentrations were determined by the Salkowski reaction (17). A Voges-Proskauer test (40) was used for detection of acetoin production. Chrome azurol S (CAS) plates (38) were used to determine siderophore secretion (orange halos around the colonies indicated siderophore excretion). Bacterial strains were grown on PKO agar medium (53) with 0.5% tricalcium phosphate as the inorganic phosphate source and on Mongina organic culture medium (20a) with lecithin as an organic phosphate source to determine the phosphorus-solubilizing activity. A clear halo around the bacterial colonies indicated the phosphate solubilization capacity of a bacterial strain. All tests were carried out in triplicate for reproducibility.

Transposon mutagenesis, screening, and sensitivity tests. Transposon insertion mutants were generated by mobilization of the suicide plasmid pRL27 (Km^r) from the donor strain *Escherichia coli* BW20767 to the recipient strain *A. tumefaciens* CCNWGS0286 (Cm^r) by biparental mating. A random insertion mutant library was generated. The mutagenized cells were plated on TY medium containing chloramphenicol (20 μg/ml) and kanamycin (50 μg/ml). Colonies resistant to both antibiotics were pooled and were then picked up onto TY plates with a final concentration of 1.8 mM Zn^{2+}. Clones that were unable to grow in the presence of 1.8 mM Zn^{2+} were recovered and were subjected to further analyses.

Whole genomic DNA of zinc-sensitive mutants of *A. tumefaciens* CCNWGS0286 obtained by the screening test was further analyzed following digestion (SacII or EcoRI), self-ligation, and electroporation into *E. coli* EC1000 pir-116. The plasmids containing the modified Tn*5* plus the adjacent region of the chromosomal DNA were isolated using the QIAprep Spin Miniprep kit (Qiagen, MD). The sequence of the adjacent chromosomal DNA was obtained using primers specific to the ends of the transposon: tpnRL17-1 (5′-AACAAGCCAGGGATGTAACG-3′) and tpnRL13-2 (5′-CAGCAACACCTTCTTCACGA-3′).

Stationary-phase cells of *A. tumefaciens* CCNWGS0286 and mutants were inoculated in TY medium supplemented with different concentrations of Zn^{2+}, Cd^{2+}, and Cu^{2+}. The initial optical density of the cell suspension at 600 nm (OD_{600}) was adjusted to around 0.02. The cultures were incubated at 30°C with agitation at 200 rpm. Samples were taken after 24 h (samples without metal ions added had reached stationary phase), and the OD_{600} was determined with a Perkin-Elmer UV-visible (UV-Vis) spectrophotometer.

Gene expression analyses of P-type ATPases and CzcD. The expression of four genes encoding putative P-type ATPases and *czcD*, encoding a member of the cation diffusion facilitator (CDF) family, was assessed by reverse transcriptase (RT) PCR using the wild type and a mutant strain with an interruption of *zntR*, encoding a regulator of the MerR family. Total RNA was extracted from mid-exponential-phase cells either left untreated or treated with 0.5 mM Zn^{2+} for 15 min in TY medium. Total RNA was isolated by the RNeasy Mini kit (Qiagen, MD) and was then treated with DNase I (Fermentas, MD) according to the manufacturer's instructions. PCR tests using total RNA as the template were used to verify the absence of DNA. Equal amounts of total RNA as determined by the OD_{260} were used for cDNA synthesis using an iScript Select cDNA synthesis kit (Bio-Rad, CA). Primers were designed to monitor the transcription of genes encoding putative P-type ATPases: these were genes 153 (ATCR1_00750), 461 (ATCR1_02265), 4200 (ATCR1_21065), and 4343 (ATCR1_21784), as well as *czcD* (ATCR1_22014) (see Table S1 in the supplemental material). The 16S rRNA gene was used as a positive control. The PCR program for all tested gene amplifications was 95°C for 5 min, followed by 32 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 6 min. PCR products were analyzed by 1% agarose gel electrophoresis.

Microbe-assisted phytoremediation. The effects of bacteria on plant growth promotion were tested in cups filled with 40 ± 1 g sterilized vermiculite either alone or supplemented with 300 or 600 mg/kg Zn. *Robinia pseudoacacia* seeds were treated in the following order: concentrated sulfuric acid (10 min), 95% ethanol (20 s), 5% sodium hypochlorite (15 min), and sterile distilled water (washing) for surface sterilization. Sterilized seeds were germinated in petri dishes with water agar at 28°C, and then seedlings were sown in cups filled with 40 ± 1 g sterilized vermiculite (46) and were incubated in the greenhouse at 25°C. After the first main leaf grew out, *A. tumefaciens* CCNWGS0286 (zinc resistant and IAA positive), *A. tumefaciens* C58 (zinc resistant and IAA negative), mutants 12-2 (zinc sensitive and IAA positive), and 15-6 (zinc sensitive and IAA negative) were inoculated with 10^8 CFU per root. Seedlings without inoculation were included as blank controls. Plants were harvested after 45 days, and the length and dry weight of shoots and roots were determined. Statistical analyses were performed with SAS software, version 8.

Genome sequencing and analysis. High-quality genomic DNA (30 to 50 μg/μl) was extracted from *A. tumefaciens* strain CCNWGS0286 using the Blood and Cell Culture DNA Mini kit (Qiagen, MD). Whole-genome shotgun sequencing was performed at the University of Arizona Genetics Core Facility by using a Roche 454 Genome Sequencer FLX instrument at 27-fold coverage. Assembly was performed using the GS *De novo* Assembler (version 2.5.3) software program. Subsystem classification and functional annotation were compiled using the Subsystem Technology RAST server (2). BLAST in the RAST server was used for resistant and metabolic gene function identification. Metabolic pathways were predicted according to the KEGG pathway map (http://www.genome.jp/).

Nucleotide sequence accession number. The draft genome sequence has been deposited in GenBank under accession number AGSM00000000. The version described in this paper is the first version, AGSM01000000.

RESULTS

***Agrobacterium tumefaciens* CCNWGS0286 promoted the growth of *Robinia pseudoacacia* under zinc stress.** The endophytic bacterium CCNWGS0286 was isolated from surface-ster-

ilized nodules of *Robinia pseudoacacia* grown in a lead-zinc mine tailing in Gansu Province in northwestern China, where the physiognomy and vegetation have been seriously damaged due to the long period of mining exploitation. The sequence of the 16S rRNA gene of strain CCNWGS0286 was 99% similar to that of *Agrobacterium tumefaciens* strain ATCC 13332 (GenBank accession no. HQ735085). Specifically, based on comparison of 16S rRNA sequences as demonstrated in a phylogenetic analysis (see Fig. S1 in the supplemental material), strain CCNWGS0286 belongs to *Agrobacterium tumefaciens* biovar I. In addition, protelomerase, which is responsible for generating the hairpin ends in the linear DNA, was reported to be present only in biovar I strains (33). A gene encoding protelomerase (ATCR1_05421) was identified in the CCNWGS0286 genome, supporting the finding that this strain belongs to *Agrobacterium tumefaciens* biovar I. The MICs (determined in TY agar) of Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Ni^{2+}, Cr^{3+}, and Cr^{6+} for this strain were 2.8, 3.0, 2.5, 0.5, 2.2, 2.6, and 1.6 mM, respectively. Plant tests showed that *A. tumefaciens* CCNWGS0286 promoted the growth of *Robinia pseudoacacia* significantly, with increases of 14.63%, 23.56%, and 28.07% in the dry weight of stems in the presence of 0, 300, and 600 mg/kg zinc, respectively, relative to growth without inoculation. The physiological and biochemical characteristics of CCNWGS0286 showed that the strain had a pH tolerance range from pH 4 to 11, tolerance to as much as 3% NaCl, resistance to the antibiotics chloromycetin (5 to 500 μg/ml), ampicillin (5 to 500 μg/ml), rifampin (5 to 300 μg/ml), kanamycin (0 to 6 μg/ml), and lincomycin (0 to 5 μg/ml), and sensitivity to streptomycin and azithromycin. It could grow in a medium supplemented with methyl orange (0.2%), Congo red (0.1%), neutral red (0.2%), or thymol blue (0.2%). Strain CCNWGS0286 could use glucose, sucrose, D-fructose, arabinose, lactose, galactose, amylomaltose, mannose, rhamnose, D-xylose, trisodium citrate, and sodium propionate as sole carbon sources and could use L-alanine, L-leucine, L-aspartate, L-arginine, L-cystine, L-tyrosine, L-methionine, DL-histidine, DL-α-lactamine, and phenylalanine as sole nitrogen sources, but it was not able to grow autotrophically and fix nitrogen.

Genes putatively conferring zinc resistance could be identified in the draft genome of *Agrobacterium tumefaciens* CCNWGS0286. Rhizosphere bacteria have to adapt in order to survive in a metal-contaminated environment. PGPB secrete metabolites, such as extracellular polysaccharide, organic acid, siderophores, lipids, and proteins, which could be used for chelation, complexation, precipitation, and adsorption of heavy metals present in the rhizophere (32). Moreover, microbes could also resist, adsorb, accumulate, oxidize, and reduce metal ions themselves, thereby helping to improve phytoremediation (26).

The draft genome sequence of *A. tumefaciens* CCNWGS0286 comprises 5,215,281 bases with a 27-fold coverage of the genome. It contains 5,013 open reading frames (ORFs), 44 tRNAs, and 3 rRNAs with an average G+C content of 59.53%. Of the 4,966 proteins, 4,110 have hits in reference strain *A. tumefaciens* C58 within the threshold of 1e−5 by BLASTP, version 2.2.24+. In addition, 4,082 proteins could be assigned to COG (*c*lusters of *o*rthologous *g*roups) families through the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). Genes putatively involved in zinc homeostasis were identified on the *A. tumefaciens* CCNWGS0286 genome; they included four genes encoding putative cation (Pb, Cd, Zn, Hg, or Cu)-transporting ATPases (ATCR1_00750, ATCR1_02265, ATCR1_21065, and ATCR1_21784) regulated by MerR-type regulators and genes encoding zinc ABC transporters (ZnuABC) (ATCR1_02230, ATCR1_02220, ATCR1_02225, ATCR1_24860, ATCR1_24865, and ATCR1_24870). The genome also encodes proteins involved in resistance to other metals, including a putative multiple copper oxidase (ATCR1_19501) possibly regulated by adjacently encoded AraC, an arsenate reductase (ATCR1_02075) regulated by ArsR (not found in C58), and chromate transport protein ChrA (ATCR1_03154 and ATCR1_19096). Compared to the genome of *A. tumefaciens* C58 (see Table S2 in the supplemental material), no additional genes except for the genes encoding metal resistance determinants mentioned above were found on the *A. tumefaciens* CCNWGS0286 genome, indicating a similar level of metal resistance, which was confirmed by MIC tests of both strains. These findings also suggested that the metal resistance genes encoded in *A. tumefaciens* CCNWGS0286 might be enough to ensure survival and the ability to compete with other microbes in the rhizosphere of plants in a metal-contaminated environment.

Transposon mutagenesis identified a P-type ATPase, a MerR-type regulator, and a gene possibly involved in lipid transport in a screen for zinc sensitivity. Among the 7,600 Tn*5* insertional mutants of *A. tumefaciens* CCNWGS0286 (strain 176), nine zinc-sensitive isolates were screened individually on TY agar medium supplemented with 1.0, 1.5, or 1.8 mM zinc. In order to identify the degrees of sensitivity and metal specificity, growth tests of the nine zinc-sensitive mutants in the presence of Zn^{2+}, Cd^{2+}, and Cu^{2+} were performed. Although the degrees of sensitivity toward different metals differed among these mutants, all mutants except for mutant 15-6 were sensitive to 0.2 mM Zn^{2+} or Cd^{2+}, with OD_{600}s much lower than that of the wild-type strain (Fig. 1A and B). However, there was no difference in sensitivity toward Cu^{2+} between eight of the mutants and the wild-type strain (Fig. 1C), indicating that the mutations specifically affected resistance to Zn^{2+} and Cd^{2+}. Although the growth of mutant strain 15-6 was delayed even without added metal, it also showed great sensitivity to 0.6 mM Zn^{2+}.

The insertion sites of individual Tn*5* transposons could be located on the whole genome of *A. tumefaciens* CCNWGS0286 (Fig. 2A). Tn*5* inserted into the same gene encoding a putative Zn(II), Cd(II), Pb(II)-transporting P-type ATPase (ATCR1_21065), subsequently named ZntA-4200, at different locations in six mutants (12-2, 46-34, 75-29, 80-15, 100-29, and 110-34). Although Tn*5* inserted into the same gene in these six mutants, different insertion locations had different effects on growth under zinc stress (Fig. 1A). In mutant 12-2, Tn*5* had inserted near the end of the gene, in a region encoding the C terminus of the P-type ATPase, which did not completely abolish ZntA-4200-mediated zinc resistance, in contrast to the other mutants (46-34, 75-29, 80-15, 100-29, and 110-34) with insertions closer to the start of the gene. Another pair of mutants, 18-12 and 19-18, were both interrupted in the same gene (ATCR1_22029) at different insert locations. This gene, encoding a putative transcriptional regulator of the MerR family subsequently named ZntR1, could have been responsible for regulating the expression of the adjacent gene *czcD*, encoding a putative cobalt-zinc-cadmium transporter of the cation diffusion facilitator (CDF) family, or one or more cation-transporting P-type ATPases. Finally, a gene encoding a putative oligoketide cyclase/lipid transport protein (ATCR1_01840) was interrupted in mutant strain 15-6.

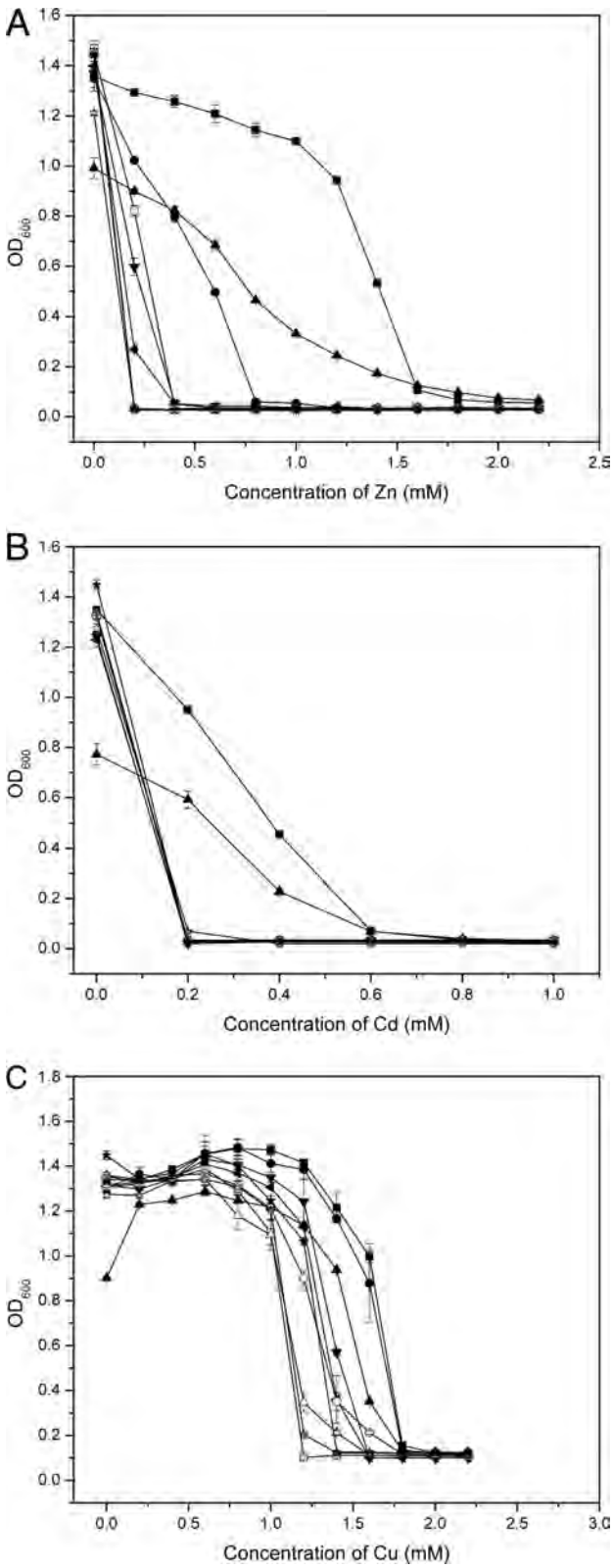

FIG 1 Growth of wild-type *A. tumefaciens* CCNWGS0286 (strain 176) and nine Tn5 insertion mutants in 0 to 2.2 mM Zn^{2+} (A), 0 to 1.0 mM Cd^{2+} (B), and 0 to 2.2 mM Cu^{2+} (C). Symbols represent the wild-type control (■) and mutants 12-2, 15-6, 18-12, 19-18, 46-34, 75-29, 80-15, 100-29, and 110-34 of *A. tumefaciens* CCNWGS0286 (strain 176) (●, ▲, ▼, ◄◄, □, ○, △, ★, and ☆, respectively). Cells grown overnight were diluted 1:100 and were then incubated at 30°C with shaking at 200 rpm. Samples at different metal concentrations were taken when the wild type in the absence of metal ions reached the stationary phase (24 h).

ZntR1 and ZntA-4200 constitute the main Zn and Cd resistance systems. In order to further characterize the most important genes responsible for zinc resistance in *A. tumefaciens* CCNWGS0286, RT-PCR was conducted with genes encoding putative P-type ATPases and *czcD* by using the wild-type strain and ZntR1$^-$ mutant 19-18 (*zntR*::Tn*5*) induced by 0.5 mM Zn^{2+} (Fig. 2B). In all cases, 16S rRNA was readily amplified, indicating that the RNA extracted from treated and untreated cells was usable and of good quality. RT-PCR products corresponding to the corrected sizes of 496 bp (P-type ATPase 461), 947 bp (P-type ATPase 4200), 988 bp (P-type ATPase 4343), and 690 bp (*czcD*) were amplified from cDNA with or without Zn treatment. ZntA-4200 was upregulated in the wild-type strain treated with Zn^{2+}, which was consistent with the result obtained by transposon mutagenesis (mutants 12-2, 46-34, 75-29, 80-15, 100-29, and 110-34). However, no ZntA-4200 expression was identified in mutant 19-18 (*zntR*::Tn*5*) when it was treated with Zn^{2+}, indicating that ZntA-4200 was regulated by ZntR1. This is in agreement with the results of transposon mutagenesis showing that mutants with interruptions of ZntA-4200 (mutants 12-2, 46-34, 75-29, 80-15, 100-29, and 110-34) and ZntR1 (mutants 18-12 and 19-18) were sensitive to 0.2 mM Zn^{2+} and Cd^{2+}. *czcD* was not induced in the presence of ZntA-4200, which ensured that intracellular levels of Zn^{2+} were kept very low. P-type ATPase CopA 461 was not induced by Zn^{2+} and is likely involved in the transport of Cu(I) into the cbb_3 cytochrome oxidase complex, since some of the genes encoding cbb_3 cytochrome oxidase are located adjacent to *copA461*. However, the expression of P-type ATPase CopA 461 was downregulated in mutant 19-18 (*zntR*::Tn*5*) under Zn^{2+} stress. Since *czcD* was induced in the *zntR1* mutant, ZntR1 and ZntA-4200 constitute the main Zn and Cd resistance systems.

Zinc-sensitive mutants of *A. tumefaciens* could still promote the growth of *R. pseudoacacia* under zinc stress. Wild-type *A. tumefaciens* CCNWGS0286 (strain 176), zinc-sensitive mutant 12-2 (*zntA*::Tn*5*) with a P-type ATPase interruption, and *A. tumefaciens* C58, with the same zinc resistance level as CCNWGS0286 (strain 176), were used to determine if the ability to tolerate higher zinc concentrations would help *Robinia pseudoacacia* survive and grow better in zinc-contaminated soil. Inoculation with either *A. tumefaciens* CCNWGS0286 (strain 176) or zinc-sensitive mutant 12-2 (*zntA*::Tn5) showed significant improvement in the growth of *Robinia pseudoacacia* (Fig. 3). In contrast to the control without inoculation (CK), there were 14.63% and 23.09% increases in the dry weight of stems and 46.13% and 54.73% increases in the dry weight of roots due to the presence of strains 176 and 12-2 (*zntA*::Tn*5*), respectively, in uncontaminated soil. Moreover, there was no significant difference in the improvement of plant growth between the two strains ($P < 0.05$) in the presence of 300 mg/kg zinc, with 23.56% and 19.89% increases found in the dry weight of stems with inoculation of strain 176 and 12-2 (*zntA*::Tn*5*) over that for the CK in the presence of 300 mg/kg zinc, indicating that genes encoding zinc resistance determinants were not responsible for stimulating plant growth in soil with 300 mg/kg zinc. Similar results were obtained when 600 mg/kg zinc was added to the soil.

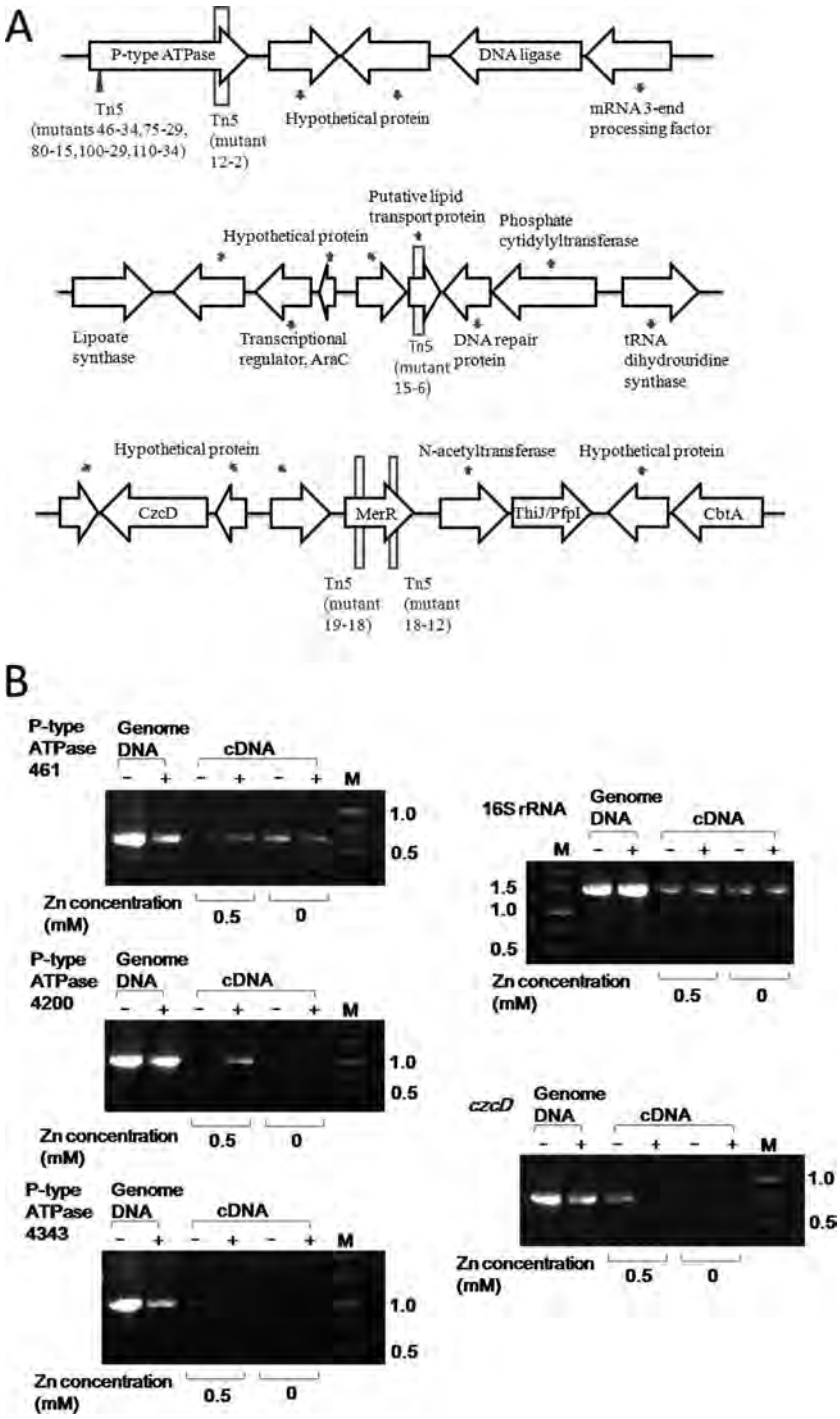

FIG 2 (A) Physical map of genes interrupted by Tn*5* related to the zinc resistance of *A. tumefaciens* CCNWGS0286. (B) Expression of *czcD* and P-type ATPases. RT-PCR products of genes encoding putative P-type ATPases and *czcD* were amplified from cDNA extracted from the wild-type strain *A. tumefaciens* CCNWGS0286 (+) and mutant strain 19-18 (*zntR*::Tn*5*) (−) induced by 0 and 0.5 mM Zn^{2+} for 15 min. 16S rRNA amplifications were included as a control. Lane M, molecular size standards (in kbp).

Therefore, the zinc-sensitive mutant *A. tumefaciens* 12-2 (*zntA*::Tn*5*) could still promote the growth of *R. pseudoacacia* under zinc stress with the same efficiency as the wild-type strain *A. tumefaciens* 176, indicating that the lack of zinc resistance had little effect on the growth of the plant under conditions of zinc stress. Although 12-2 (*zntA*::Tn*5*) did not completely lose zinc resistance, the Zn resistance of 12-2 (*zntA*::Tn*5*) was much lower than that of *A. tumefaciens* C58.

However, the positive effect of 12-2 (*zntA*::Tn*5*) on plant growth was much more significant than that of C58 ($P < 0.05$). Moreover, zinc-resistant strain C58 did not assist the growth of the plant compared to the CK no matter whether zinc was added to the soil or not

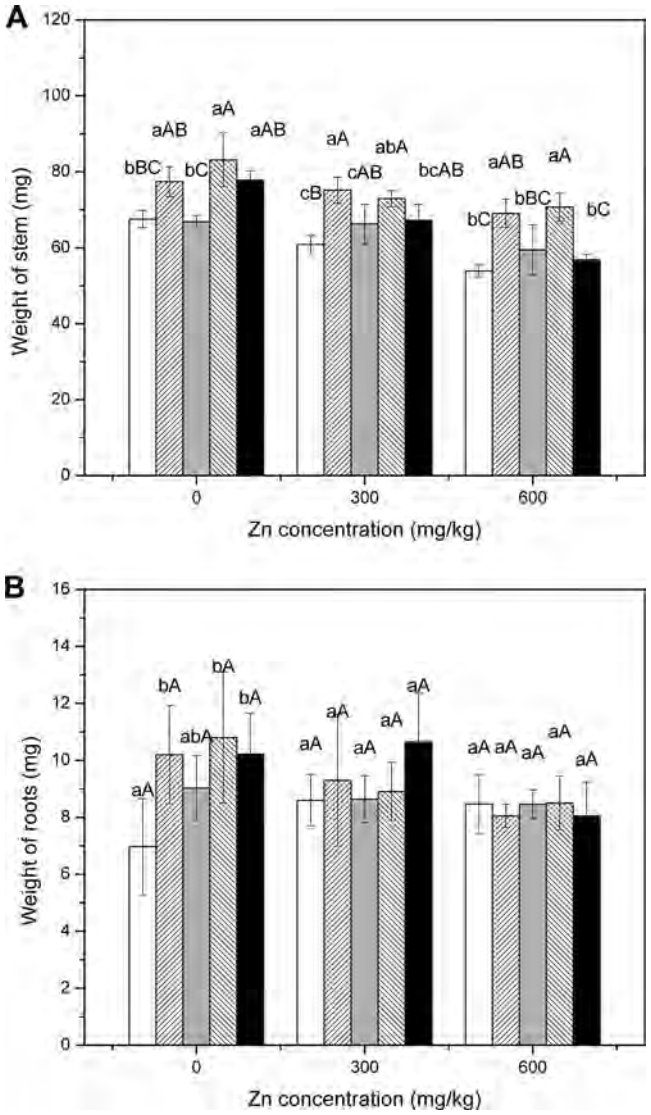

FIG 3 Influence of inoculation of *A. tumefaciens* CCNWGS0286 (strain 176) (zinc resistant, IAA overproducing), mutant 12-2 (*zntA*::Tn*5*) (zinc sensitive, IAA overproducing), mutant 15-6 (zinc sensitive, producing low levels of IAA), and *A. tumefaciens* C58 (zinc resistant but producing low levels of IAA) on the shoot growth (A) and root growth (B) of *Robinia pseudoacacia* in the presence of 0, 300, or 600 mg/kg zinc over 45 days. CK (□) was the control without inoculation of a strain. At each concentration, the second to fifth bars represent the inoculation of *A. tumefaciens* CCNWGS0286 (strain 176), C58, and mutants 12-2 and 15-6, respectively. Data labeled with different capital letters are extremely significantly different ($P < 0.01$), and those labeled with different lowercase letters are significantly different ($P < 0.05$), between treatments according to Fisher's protected least-significant-difference test.

(Fig. 3). This confirmed that the presence of genes encoding zinc resistance determinants did not aid plant growth.

Analysis of factors involved in plant growth promotion showed that IAA was overproduced in *A. tumefaciens* CCNWGS0286. *A. tumefaciens* CCNWGS0286 was found to have beneficial effects on *Robinia pseudoacacia* in a metal-contaminated environment. Although *A. tumefaciens* CCNWGS0286 was isolated from nodules of *R. pseudoacacia*, unlike rhizobia and other nitrogen-fixing bacteria, it could not form nodules with its host plant *Robinia pseudoacacia* regardless of the presence of zinc (see Fig. S2 in the supplemental material), nor could it fix nitrogen symbiotically, due to the lack of *nod* and *nif* genes in the genome (see Table S3 in the supplemental material). This was confirmed by the fact that no *nodA* or *nifH* amplicons were detected, and the strain could not grow autotrophically and fix nitrogen (25). Other plant-growth-promoting traits, such as acetoin production, ACC deaminase activity, and mineral and organic P solubilization abilities, could not be detected in *A. tumefaciens* CCNWGS0286. However, compared to other previously reported PGPB (Table 1), IAA was overproduced (10 to 20 times) in *A. tumefaciens* CCNWGS0286 (Fig. 4A). Statistical analyses showed overproduction of IAA by *A. tumefaciens* CCNWGS0286 (43.07 ± 0.95 mg/liter) compared to reference strain C58 (2.94 ± 0.31 mg/liter). There was no significant difference ($P < 0.01$) between wild-type strain 176 and the various mutant strains except for 15-6. In mutant strain 15-6, in which a gene encoding a putative oligoketide cyclase/lipid transport protein was interrupted, the production of IAA decreased dramatically to 9.31 ± 0.63 mg/liter. However, the IAA production of 15-6 was still higher than the IAA production of reference strain C58 ($P < 0.01$).

Various rhizobacteria have been reported to benefit their host plants in heavy-metal-contaminated environments (22, 24, 44). Dimkpa et al. showed that the production of phytohormones could still proceed under heavy-metal stress (10, 11). However, which factor—the presence of genes conferring resistance to metals in these rhizobacteria or increased production of phytohormones—played the dominant role for the survival of the plant in a metal-contaminated environment was not known. In order to gain further understanding, we first examined the influence of metals on the production of phytohormones. Figure 4B shows the effects of different concentrations of copper and zinc on IAA production by *A. tumefaciens* CCNWGS0286 (strain 176) and C58. In strain CCNWGS0286, IAA production kept stable under 0.5 mM Zn^{2+} at 43.07 ± 0.95 mg/liter and decreased dramatically to about half when the Zn^{2+} concentration increased to 1.0 mM. Interestingly, IAA production remained at around 20 mg/liter when Zn^{2+} concentrations were further increased from 1.0 to 2.0 mM, a level that was still much higher than the production by C58 without zinc. In comparison, Cu^{2+} seemed to have a much greater effect on IAA production. When the Cu^{2+} concentration increased to 1.0 mM, almost no IAA was produced any more.

Overall, *A. tumefaciens* strain CCNWGS0286 could significantly overproduce IAA compared to reference strain C58. All zinc-sensitive mutants could produce the same level of IAA as the wild-type strain except for mutant 15-6. Although the IAA production capacity of zinc-sensitive mutant 15-6 was dramatically affected by the insertion of Tn*5*, its IAA production was still higher than that of C58.

Phytohormones were the dominant factor for the growth of *Robinia pseudoacacia* in zinc-contaminated soil. Through transposon mutation and the analysis of the effect of heavy metals on IAA production, wild-type strain CCNWGS0286 (strain 176) (zinc resistant, IAA overproducing), mutant 12-2 (zinc sensitive, IAA overproducing), mutant 15-6 (zinc sensitive, with moderate IAA production), and reference strain C58 (zinc resistant but with low IAA production) were chosen for the determination of the dominant factor for the growth of *Robinia pseudoacacia* in zinc-contaminated soil. *A. tumefaciens* CCNWGS0286 (strain 176) and mutant 15-6 conferred significant improvement on the growth of *Robinia pseudoacacia* (Fig. 3). In contrast to the control without

TABLE 1 Examples of IAA production by PGPB from metal-contaminated environments

PGPB strain	Host plant for expt	Source of bacteria	Metal resistance[a]	IAA production (mg/liter)[b]	Other promoting factors	Effect on host[c]	Reference
Pseudomonas sp. strain SRS8	*Brassica juncea*, *Brassica oxyrrhina*	Rhizosphere of *Alyssum serpyllifolium* at a serpentine site	1,000 mg/liter Ni	111 (±1)	Phosphate solubilization, siderophore production	15.45% Increase in *B. juncea* and 17.5% increase in *B. oxyrrhina* under 450 mg/kg Ni	28
Pseudomonas sp. strain PsM6	*Ricinus communis*	Serpentine soil	800 mg/liter Ni, 750 mg/liter Cu, 700 mg/liter Zn	17.74 (±2.06)	ACC deaminase, phosphate solubilization	15% Increase in shoot wt and 18% increase in root wt in metal-contaminated soil; increased Cu and Zn accumulation in plant tissues	35
Pseudomonas jessenii PjM15	*Ricinus communis*	Serpentine soil	900 mg/liter Ni, 600 mg/liter Cu, 700 mg/liter Zn	39.88 (±3.68)	ACC deaminase, phosphate solubilization	20% Increase in shoot wt and 25% increase in root wt in metal-contaminated soil; increased Cu, Zn, and Ni accumulation in plant tissues	35
Enterobacter sp. strain NBRI K28	*Brassica juncea*	Fly ash-contaminated region	—	41.8 (±0.6) mg/kg	ACC deaminase, phosphate solubilization, siderophore production	Increased Ni and Cr accumulation in *B. juncea* with 25% FA amendments	23
Enterobacter sp. strain SC20	Sugarcane	Sugarcane root	—	0.75	Nitrogen fixation	55% Increase in root wt and 70% increase in shoot wt	37
Bacillus sp. strain SRI14	*Brassica juncea*, *Brassica oxyrrhina*	Rhizosphere of *Astragalus incanus* at a serpentine site	750 mg/liter Ni	20 (±1)	ACC deaminase, phosphate solubilization, siderophore production	11.4% Dry wt increase in *B. juncea* and 8.75% dry wt increase in *B. oxyrrhina* under 450 mg/kg Ni	28
Achromobacter xylosoxidans Ax10	*Brassica juncea*	Cu mining	600 mg/liter Cu	6.4	Inorganic phosphate solubilization	Increased shoot and root length, wt, and Cu accumulation with 50 and 100 mg/kg Cu	27
Serratia marcescens BR780	*Salix caprea*	Lead mining	8 mM Zn, 4 mM Cd, 2 mM Pb	NQ	Siderophore production	No effect on Zn and Cd uptake by *Salix caprea*	22
Burkholderia sp. strain D54	*Sedum alfredii*	Metal-contaminated soils in mining area	2,000 mg/liter Cd, 800 mg/liter Pb, 150 mg/liter Cu, 2,500 mg/liter Zn	0.7	ACC deaminase, phosphate solubilization, siderophore production, insoluble metal solubilization	Enhanced plant biomass and increase total shoot and root Cd, Pb, and Zn uptake (mg per pot) in *S. alfredii*	18
Sinorhizobium meliloti CCNWSX0020	*Medicago lupulina*	Mine tailings	1.4 mM Cu	13.8 (±1.3)	Nitrogen fixation	78.2% Increase in biomass with 100 mg/kg Cu and 39.3% increase in Cu accumulation in plant tissues	13
Rhizobium leguminosarum bv. *trifolii*	*Oryza sativa* L.	Sterilized rice root	—	2.5 (±0.5)	Nitrogen fixation	Increased shoot and root growth of rice	34, 52
Rhizobium sp.	*Vigna mungo* (L.)	Root nodule	—	28.33	Nitrogen fixation	Physiological implications in nodulation of plant	29
Agrobacterium tumefaciens C58	*Robinia pseudoacacia*		2.8 M Cu, 2.6 mM Zn	2.94 (±0.31)	None	No significant enhancement for plant	This study
Agrobacterium tumefaciens CCNWGS0286	*Robinia pseudoacacia*	Root nodule, zinc-lead mine tailing	2.8 mM Cu, 3.0 mM Zn, 2.5 mM Pb, 0.5 mM Cd, 2.2 mM Ni, 2.6 mM Cr(III), 1.6 mM Cr(IV)	43.07 (±0.95)	None	23.56% and 28.07% increases in dry stem wt under 300 and 600 mg/kg zinc, respectively	This study

[a] —, no data detected.
[b] NQ, data were detected but could not be quantified.
[c] FA, fly ash.

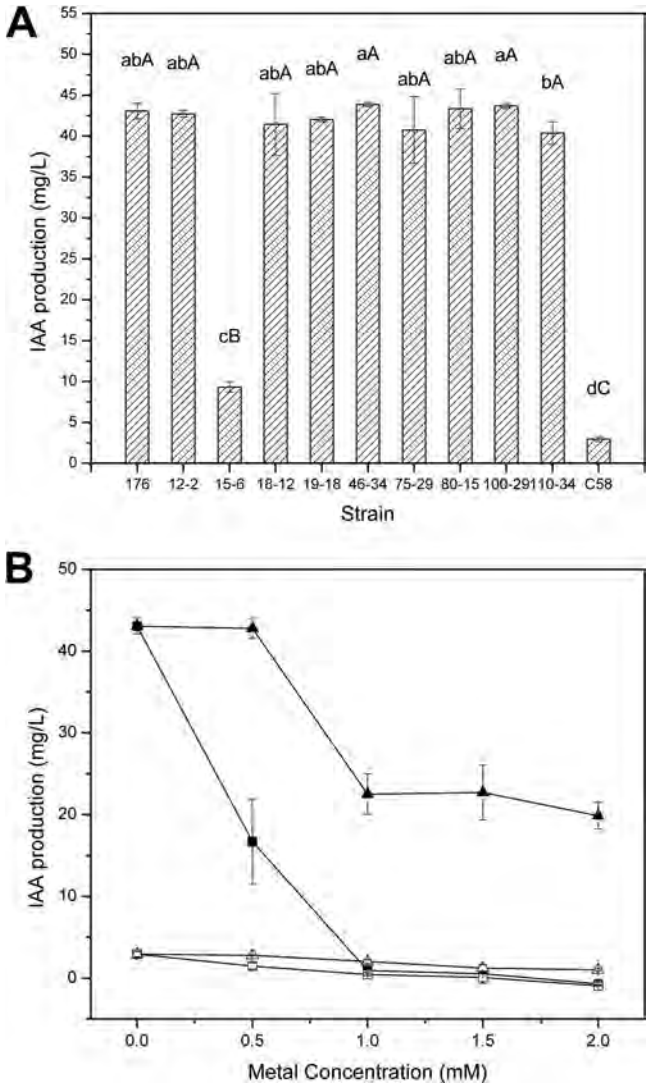

FIG 4 Production of IAA (mg/liter) under different conditions. (A) IAA production (mg/liter) by *A. tumefaciens* CCNWGS0286 (strain 176), mutant strains, and reference strain *A. tumefaciens* C58 without metal stress. (B) Effects of different Zn^{2+} and Cu^{2+} concentrations on the production of IAA by *A. tumefaciens* CCNWGS0286 (strain 176) and C58. ■ and □, IAA production by *A. tumefaciens* CCNWGS0286 (strain 176) and C58, respectively, in the presence of copper. ▲ and △, IAA production by *A. tumefaciens* CCNWGS 0286 (strain 176) and C58, respectively, in the presence of zinc. Data labeled with different capital letters are extremely significantly different ($P < 0.01$), and those labeled with different lowercase letters are significantly different ($P < 0.05$), between treatments according to Fisher's protected least-significant-difference test.

inoculation (CK), there were 14.63% and 15.21% increases in the dry weight of stems and 46.13% and 46.28% increases in the dry weight of roots inoculated with strain 176 or 15-6 in non-zinc-contaminated soil, respectively. However, a significant difference in plant biomass could be observed between strains 176 and 15-6 when 300 or 600 mg/kg zinc was added ($P < 0.05$). Mutant 15-6 could still promote plant growth (10.39%) under an intermediate zinc stress of 300 mg/kg, but much less so than other strains (a 23.56% increase for strain 176 and a 19.89% increase for 12-2 [*zntA*::Tn*5*]). In contrast, when high zinc levels of 600 mg/kg were reached, no difference in plant biomass was found between inoculation with 15-6 and the CK, suggesting that 15-6 could not promote plant growth at very high zinc concentrations.

Overall, the wild-type strain 176 and the zinc-sensitive mutant strain 12-2 (*zntA*::Tn*5*), both with upregulated IAA production, could help the plant withstand the inhibitory effect of elevated zinc concentrations. Zinc-resistant strain C58 did not help the plant to grow no matter whether zinc was added or not. In contrast, the zinc-sensitive, low-level-IAA-producing mutant 15-6 did help the plant to grow in the absence of zinc at the same level as the wild type ($P < 0.05$). However, 15-6 could not promote plant growth at very high zinc concentrations, because the cells of 15-6 were dying under zinc stress. All these data analyzed above demonstrated the ability of *A. tumefaciens* CCNWGS0286 (strain 176) to enhance plant biomass and overcome the inhibition caused by the presence of zinc in the soil. Additionally, in the system we studied, the beneficial effect of IAA had a much greater influence than the presence of metal resistance determinants in CCNWGS0286 for plant growth enhancement in a metal-contaminated environment.

DISCUSSION

Agrobacterium tumefaciens is a ubiquitous soil bacterium, virulent strains of which can cause disease in more than 90 families of dicotyledonous plants (51). However, not all *A. tumefaciens* strains are pathogenic; some are beneficial and assist rhizobia with legume nodulation (8, 25, 31). The plant-growth-promoting endophytic bacterium *A. tumefaciens* CCNWGS0286, isolated from zinc-lead mine tailings, displayed both high metal resistance and the ability to enhance the growth of *Robinia pseudoacacia* in a metal-contaminated environment. Genomic analysis, physiological studies, and biochemical experiments showed a strong capacity of *A. tumefaciens* CCNWGS0286 to use various carbon sources and amino acids, including glucose, sucrose, fructose, arabinose, mannose, and D-xylose, most of which are plant secreted and indicate an adaptation to plant hosts. Probable chromosomal and plasmid-borne virulence genes were present in the genome of *A. tumefaciens* strain CCNWGS0286, but neither a complete *vir* gene system nor classic tumor-inducing T-DNA genes were present in this strain (see Table S4 in the supplemental material).

A number of transition metals (Cu, Zn, Ni, Mn, Fe) are micronutrients necessary for various metabolic pathways during the plant growth process (24), but high levels of metal(loid)s are toxic to plants (45). Therefore, with an increasing number of plant-growth-promoting bacteria (PGPB) reported, bacterially assisted phytoremediation in heavy-metal-contaminated environment deserves special attention (9, 39, 53). The presence of genes conferring heavy-metal resistance in microbes provides a selective advantage for survival in contaminated environments (42). In addition, phytohormones produced by microbes also play an important role in strengthening plant growth in applications such as phytoremediation (4). We assumed that the beneficial contributions of these bacteria to the plants in a metal-contaminated environment would be due either to the presence of metal resistance determinants or to plant-growth-promoting factors. However, there were no previous reports comparing the effects of these two factors on the enhancement of plant growth and determining which factor is more important in a metal-contaminated environment.

One the one hand, *A. tumefaciens* CCNWGS0286 possesses a

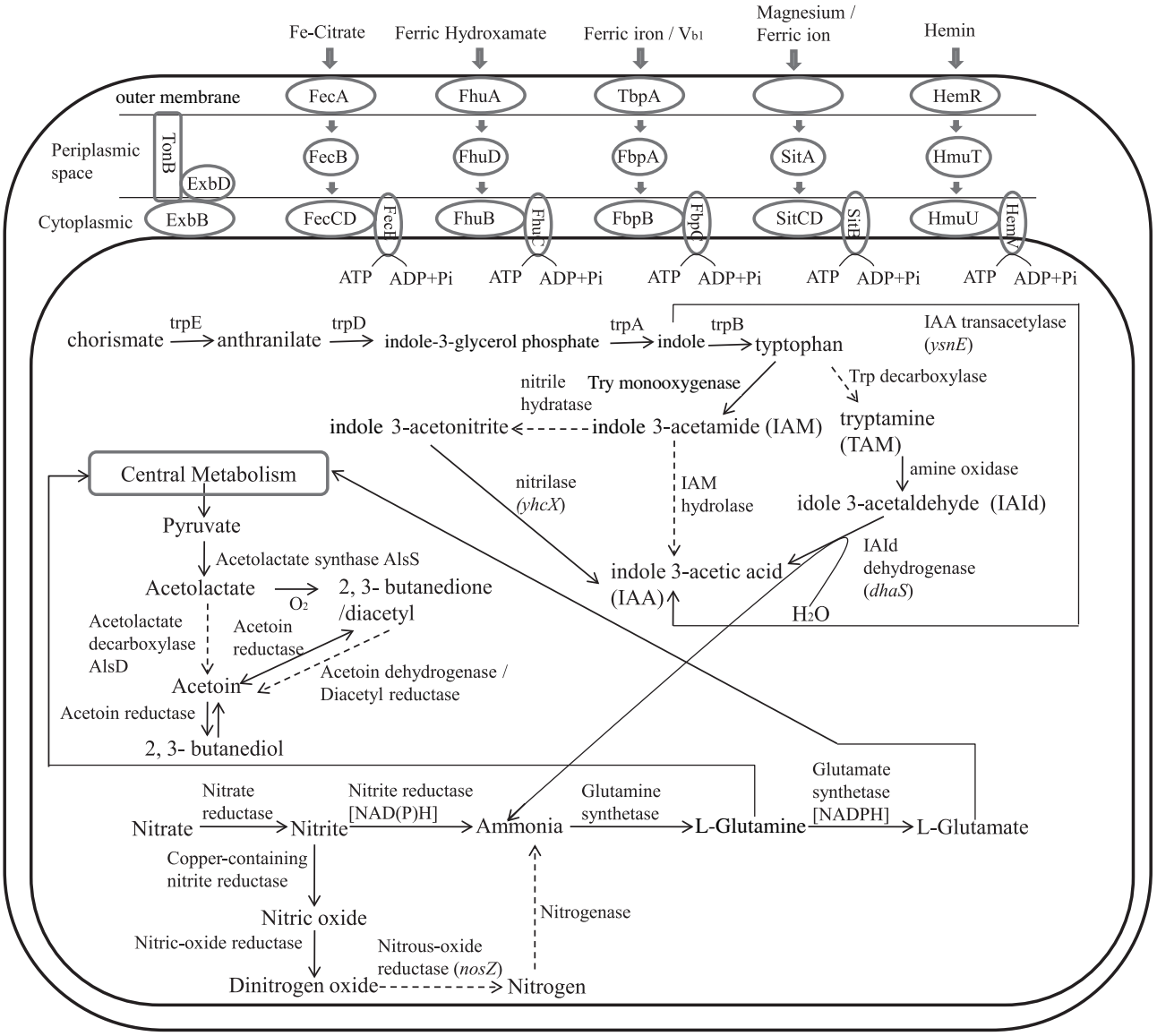

FIG 5 Probable metabolism and transport pathways identified in the genome of *A. tumefaciens* CCNWGS0286 that are involved in the promotion of the growth of *R. pseudoacacia*. The arrows show the putative direction of synthesis or transport pathways that could be identified in the genome. Dashed arrows represent known steps in which necessary enzymes are involved but that could not be identified in the genome. For IAA synthesis, two pathways, the tryptamine (TAM) pathway and the indole-3-acetamide (IAM) pathway, were predicted in *A. tumefaciens* CCNWGS0286. *trpEDABC* (ATCR1_06021 and ATCR1_10873, ATCR1_02965, ATCR1_10883, ATCR1_10878, ATCR1_02970, and ATCR1_23021) were involved in tryptophan biosynthesis initially. In the TAM pathway, genes encoding amine oxidase (ATCR1_17572) and indole-3-acetaldehyde dehydrogenase (*dhaS*) (ATCR1_06371) were identified. In the IAM pathway, putative nitrilase (carbon-nitrogen hydrolase) (*yhcX*) (ATCR1_17003) and monooxygenase (ATCR1_07569) were identified. However, genes encoding enzymes such as Trp decarboxylase in the TAM pathway and IAM hydrolase in the IAM pathway were absent. It has been proved that *ysnE* knockout would lead to diminished IAA production (19). Combined with the incomplete pathways described above, IAA transacetylase (*ysnE*) (ATCR1_18675), used for catalyzing indole to IAA, was thought to play an important role in IAA biosynthesis by *A. tumefaciens* CCNWGS0286. For acetoin and 2,3-butanediol synthesis, *poxB* like-genes (ATCR1_01785 and ATCR1_01790) encoding pyruvate dehydrogenase, used for pyruvate conversion, were found. Acetolactate synthase (AlsS) (ATCR1_17262, ATCR1_17267) could convert two pyruvate molecules to acetolactate using thiamine pyrophosphate (6). However, genes related to acetolactate decarboxylase (AlsD) and acetoin dehydrogenase/diacetyl reductase were absent in the *A. tumefaciens* genome, which means that acetoin could not be produced either by converting acetolactate to acetoin (AlsD) or by reducing 2,3-butanedione to acetoin by acetoin dehydrogenase. However, the presence of acetoin reductase (AR)/2,3-butanediol dehydrogenase (BDH) (ATCR1_12433) could aid in the production of acetoin from diacetyl. In addition, AR/BDH (ATCR1_12433) should reduce existing acetoin to 2,3-butanediol. Siderophore systems included the ferric ion ABC transporter system SitABCD (ATCR1_11128, ATCR1_11123, ATCR1_11118, ATCR1_11113) for iron uptake, the hydroxamate ABC transporter FhuABCD (ATCR1_19791, ATCR1_19801, ATCR1_19806, ATCR1_19796) for ferrichrome uptake, the hemin ABC transporter HemTUV (ATCR1_05771, ATCR1_05776, ATCR1_05781), and the Ton system TonB-ExbBD (ATCR1_16623, ATCR1_18020) for binding and transporting iron-loaded siderophores to the periplasmic space (14). The ferric citrate system FecABCDE (*fecA*, ATCR1_15296; *fecB*, ATCR1_08099, ATCR1_05711, ATCR1_05686; *fecCD*, ATCR1_08094, ATCR1_08089, ATCR1_08104, and ATCR1_15316; *fecE*, ATCR1_08084, ATCR1_15311, ATCR1_05671) which is well studied in *E. coli* (1, 12), also appears to play an important role in the ferric transport of *A. tumefaciens* CCNWGS028.

transition and heavy-metal regulatory and efflux network for survival. The results of transposon mutagenesis showed that interruption of *zntA-4200*, encoding the most important Zn(II)-translocating P-type ATPase, ZntA-4200 (ATCR1_21065), and *zntR1*, encoding the transcriptional regulator ZntR1 (ATCR1_22029), decreased the Zn and Cd resistance of *A. tumefaciens* CCNWGS 0286 dramatically. Combining these results with RT-PCR analysis, we could conclude that ZntR1 and ZntA-4200 were the primary systems for Zn and Cd resistance. Specifically, c*zcD* was upregulated in the ZntR1-interrupted mutant because when ZntA-4200 was not expressed, the increased intracellular Zn level led to increased transcription of *czcD*. In addition, an insertion in a gene encoding a putative oligoketide cyclase/lipid transport protein (mutant 15-6) also had a negative effect on zinc and cadmium resistance but no effect on copper resistance. Interestingly, the interruption of this gene also resulted in a decrease in IAA synthesis. However, the molecular mechanisms by which this gene influences Zn/Cd resistance and IAA synthesis are not clear yet. Almost all metal resistance-related genes contained in the *A. tumefaciens* CCNWGS0286 genome could also be found in *A. tumefaciens* C58, so there is not much difference in metal sensitivity between the two strains.

On the other hand, PGPB can actively promote plant growth mainly through nitrogen fixation, ACC deaminase, phytohormones, siderophore production, and phosphate solubilization (26). However, *A. tumefaciens* CCNWGS0286 cannot fix nitrogen, and genome analysis of *A. tumefaciens* CCNWGS0286 predicted that the pathway for metabolizing ACC was also lacking (see the supplemental materials). A Voges-Proskauer test did not detect acetoin production due to degradation of acetoin by acetoin reductase (ATCR1_12433). Genome analysis indicated the presence of efficient iron transport systems, including various ferric iron and siderophore ABC transporters, iron outer membrane receptors for iron siderophores, ferrichrome-iron, or ferric hydroxamate, and the ferric citrate (FecABCDE) and Ton systems (Fig. 5). However, no significant differences in iron transport systems were found by comparing the *A. tumefaciens* CCNWGS0286 and C58 genomes. In contrast, *A. tumefaciens* CCNWGS0286 could overproduce IAA (43.07 $\pm$ 0.95 mg/liter); its IAA production was $>$10 times higher than that of C58. In addition, the IAA production of CCNWGS0286, even under zinc-contaminated conditions, was still much higher than the IAA production of C58 in an environment without zinc contamination. Tryptamine (TAM) and indole-3-acetamide (IAM) pathways were predicted to be involved in the production of IAA by *A. tumefaciens* CCNWGS0286. Moreover, conversion from indole to IAA, catalyzed by IAA acetyltransferase (ATCR1_18675), may play an important role in IAA biosynthesis by *A. tumefaciens* CCNWGS0286 (Fig. 5).

In order to better understand the effects of both zinc resistance and phytohormone production by *A. tumefaciens* CCNWGS0286 in assisting plants to survive in contaminated environments, zinc-sensitive mutants obtained through transposon mutagenesis, together with the reference strain C58, were used to test the ability to promote plant growth in zinc-contaminated soil. We found that *A. tumefaciens* C58, with a similar level of zinc resistance but lower IAA production than *A. tumefaciens* CCNWGS0286, had no significant effect on plant survival in zinc-contaminated soil, but the wild-type strain *A. tumefaciens* CCNWGS0286 improved the growth of plants significantly in the presence of 300 mg/kg and 600 mg/kg zinc. This suggested zinc resistance of the strain did not assist the plant in a zinc-contaminated environment. Comparison of the improvement of plant biomass by CCNWGS0286 (strain 176) (zinc resistant, IAA overproducing), mutant 12-2 (*zntA*::Tn*5*) (zinc sensitive, IAA overproducing), and mutant 15-6 (zinc sensitive, low IAA producing) showed that both strains 176 and 12-2 (*zntA*::Tn*5*) could increase plant growth significantly ($P < 0.05$), by around 23.56% and 31.22%, over that with the CK (the control without inoculation) in the presence of 300 mg/kg and 600 mg/kg zinc, respectively. This result again confirmed the minor effect of bacterial metal resistance genes on plant growth promotion in a zinc-contaminated environment. Moreover, no significant change in plant biomass was found for 15-6, with lowered IAA production. Therefore, the primary metal resistance genes in *A. tumefaciens* CCNWGS0286 did not have an influence on the inhibition of plant growth by zinc. Phytohormones had a dominant role in enhancing plant growth in zinc-contaminated soil.

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