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Agrobacterium tumefaciens Zur Regulates the High-Affinity Zinc Uptake System TroCBA and the Putative Metal Chaperone YciC, along with ZinT and ZnuABC, for Survival under Zinc-Limiting Conditions

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ABSTRACT

***Agrobacterium tumefaciens* has a cluster of genes (Atu3178, Atu3179, and Atu3180) encoding an ABC-type transporter, here named *troA*, *troB*, and *troC*, respectively, which is shown here to be a zinc-specific uptake system. Reverse transcription (RT)-PCR analysis confirmed that *troA*, *troB*, and *troC* are cotranscribed, with *troC* as the first gene of the operon. The *yciC* (Atu3181) gene is transcribed in the opposite orientation to that of the *troCBA* operon and belongs to a metal-binding GTPase family. Expression of *troCBA* and *yciC* was inducible under zinc-limiting conditions and was controlled by the zinc uptake regulator, Zur. Compared to the wild type, the mutant strain lacking *troC* was hypersensitive to a metal chelator, EDTA, and the phenotype could be rescued by the addition of zinc, while the strain with a single *yciC* mutation showed no phenotype. However, *yciC* was important for survival under zinc limitation when either *troC* or *zinT* was inactivated. The periplasmic zinc-binding protein, ZinT, could not function when TroC was inactivated, suggesting that ZinT may interact with TroCBA in zinc uptake. Unlike many other bacteria, the ABC-type transporter ZnuABC was not the major zinc uptake system in *A. tumefaciens*. However, the important role of *A. tumefaciens* ZnuABC was revealed when TroCBA was impaired. The strain containing double mutations in the *znuA* and *troC* genes exhibited a growth defect in minimal medium. *A. tumefaciens* requires cooperation of zinc uptake systems and zinc chaperones, including TroCBA, ZnuABC, ZinT, and YciC, for survival under a wide range of zinc-limiting conditions.**

IMPORTANCE

Both host and pathogen battle over access to essential metals, including zinc. In low-zinc environments, physiological responses that make it possible to acquire enough zinc are important for bacterial survival and could determine the outcome of host-pathogen interactions. *A. tumefaciens* was found to operate a novel pathway for zinc uptake in which ZinT functions in concert with the high-affinity zinc importer TroCBA.

Zinc is an essential metal for bacteria because it is required for the functions of many enzymes and proteins (1, 2). However, zinc overload is toxic to cells (3–7). Bacteria have mechanisms to maintain zinc homeostasis via the coordinated response of genes involved in zinc uptake, efflux, and storage (8–13). The zinc uptake regulator Zur is a transcriptional regulator belonging to the Fur family and functions as a repressor of zinc uptake genes, including *znuABC* and *zinT* (10). To prevent excessive amounts of zinc in cells under high-zinc conditions, Zur uses Zn^{2+} as its cofactor to bind to a conserved AT-rich sequence, called the Zur box (14), found in the promoter region of the zinc uptake genes, leading to inhibition of gene expression (15–17). ZnuA is a periplasmic protein that binds zinc and transfers it to the membrane permease ZnuB and the ATPase ZnuC (15). The ZinT protein is a periplasmic zinc-binding protein (18–21) that has been shown to directly interact with and assist ZnuABC in transporting zinc in *Salmonella enterica* (22, 23).

Agrobacterium tumefaciens is an alphaproteobacterium that causes crown gall tumor in plants (24). Negative regulation of zinc uptake genes, such as *znuABC* and *zinT*, by *A. tumefaciens* Zur (Zur_{At}) has been reported previously (25). Expression of *A. tumefaciens znuABC* and *zinT* was inducible with zinc depletion and was repressed in response to increased zinc concentrations (25). Loss of Zur_{At} led to derepression of the *znuABC* and *zinT* genes and increased accumulation of intracellular zinc content (25). The roles of the periplasmic zinc-binding proteins *A. tumefaciens* ZnuA and ZinT ($ZnuA_{At}$ and $ZinT_{At}$, respectively) have been in-

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vestigated. It was found that $ZinT_{At}$ played an important role in *A. tumefaciens* survival under severe zinc shortage, whereas $ZnuA_{At}$ did not show an apparent role under the tested conditions (25). Disruption of both $znuA_{At}$ and $zinT_{At}$ slightly affected the total cellular zinc content (25), implying the existence of other, unidentified zinc uptake genes in *A. tumefaciens*.

The *A. tumefaciens* C58 genome contains a cluster of genes consisting of Atu3178, Atu3179, and Atu3180 that are annotated as a putative zinc/manganese ABC transport system (http://www.genome.jp/kegg-bin/show_organism?org=atu). Atu3178 encodes a periplasmic substrate-binding protein belonging to the TroA (transport-related operon) superfamily. Atu3179 encodes a permease, and Atu3180 encodes an ATP-binding protein. Here, we have named these genes *troA*, *troB*, and *troC* (the first gene of the operon), respectively. Atu3181 is a gene that has a transcription orientation opposite to that of the *troCBA* operon and encodes a putative metal chaperone, YciC, belonging to the COG0523 family of GTPases (26). In *Bacillus subtilis*, YciC was proposed to be a zinc chaperone that participates in an unidentified low-affinity zinc transport pathway (27, 28). *B. subtilis yciC* is regulated by Zur (28). The *A. tumefaciens troCBA* operon and *yciC* were predicted to be controlled by Zur due to the presence of a Zur box in their promoter regions (26). However, their physiological functions and metal regulation have not been experimentally verified.

The role of TroABCD in zinc transport was first reported in *Treponema pallidum* (29, 30). TroA is a substrate-binding protein, and TroB is an ATPase, while TroC and TroD form a heterodimeric cytoplasmic membrane permease. *T. pallidum* has the ZnuABC and TroABCD systems for zinc uptake (30). While the *T. pallidum* ZnuABC transporter is specific to zinc, *T. pallidum* TroABCD can transport zinc, manganese, and iron (29, 30). *T. pallidum troABCD* was shown to be negatively regulated by a zinc-responsive transcriptional regulator, TroR, a DtxR-like repressor (29). However, a later study showed that *T. pallidum* TroR is a manganese-dependent rather than a zinc-dependent regulator (31). Unlike many other bacteria, *T. pallidum* does not contain Zur, and the regulator of *T. pallidum znuABC* has not been reported.

Both ZnuA and TroA are substrate-binding proteins that belong to the cluster A-1 family (32). While ZnuA has been shown to respond specifically to zinc, the metal selectivity of TroA proteins and regulation of *tro* operons are different among bacteria (29, 33–36). *Streptococcus suis* TroA is involved in the uptake of manganese, not zinc (36), even though it can bind both Mn^{2+} and Zn^{2+} with high affinity (35). A gene encoding a protein that has high similarity to the manganese-responsive transcriptional regulator ScaR, located directly downstream of the *S. suis troA* gene, may be involved in the manganese regulation of the *troA* gene (36). *Treponema denticola troABCD* is negatively regulated by Mn^{2+} and Fe^{2+} via TroR (33). In contrast, *Corynebacterium diphtheriae troA* is under the control of Zur in a zinc-dependent manner (34).

Here, the physiological functions of the *A. tumefaciens troCBA* operon and *yciC* are characterized. The metal regulation of *A. tumefaciens troCBA* and *yciC* was investigated, and their regulator was identified. Important roles for *A. tumefaciens* TroCBA and YciC in zinc acquisition under zinc-limiting conditions were demonstrated. *A. tumefaciens* ZinT may interact with the TroCBA system. In addition, a role for *A. tumefaciens* ZnuA under zinc starvation conditions was revealed when the TroCBA system was disrupted. Our findings provide a step toward understanding how *A. tumefaciens* controls zinc homeostasis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. The growth conditions, media, and antibiotic concentrations that were used for *A. tumefaciens* and *E. coli* were reported previously (25, 44).

Molecular techniques. General molecular techniques were performed according to standard protocols (46). The nucleotide sequence of the cloned DNA fragment was confirmed by DNA sequencing (Macrogen). Plasmids were transferred into *A. tumefaciens* by electroporation (47). All the *A. tumefaciens* mutant strains were confirmed by Southern blotting.

Construction of the *A. tumefaciens troC* mutant and *yciC* mutant strains. The *A. tumefaciens troC* and *yciC* mutant strains were constructed using a single homologous-recombination method as described previously (48). The internal coding regions of *troC* (BT3751 [5′-TGAAACC GCTCGGCGGTGAG-3′] and BT3752 [5′-CTGCGCATCCTGCAACAT GG-3′]; 293 bp) and *yciC* (BT5027 [5′-GTGATGGCGACAACCGCACC-3′] and BT5028 [5′-GCCCTGGCGAAATCGAAGCG-3′]; 223 bp) were amplified by PCR using the specific primers indicated. The PCR products were cloned into the unique SmaI sites of pKNOCK-Gm and pKNOCK-Km, respectively (40). The resulting plasmids, pKNOCKTROC and pKNOCKmYCIC, were electroporated into wild-type (WT) NTL4 (a Ti plasmid-cured derivative of strain C58 with an internal deletion of the *tetA-tetR* locus) (37). The *troC* mutant (TC142) and *yciC* mutant (YC154) were selected on Luria-Bertani (LB) agar plates containing 60 μg/ml gentamicin (Gm) and 30 μg/ml kanamycin (Km), respectively.

Construction of the double-mutant (TCYC15, ZAYC15, ZTYC15, and ZURYC15) strains. The plasmid pKNOCKmZNUA (25) was electroporated into the TC142 strain, generating the TCZA15 strain (with disruptions of both the *troC* and *znuA* genes), which was selected on LB agar (LA) plates containing 60 μg/ml Gm and 30 μg/ml Km. The plasmid pKNOCKmZINT was constructed by PCR amplification of the internal coding region of *zinT* (238 bp) using primers BT3747 (5′-TGACGGA CTGGGAAGGCGAC-3′) and BT3748 (5′-ATCTCCTGGCCGTCGCT GAC-3′), which was then cloned into SmaI-digested pKNOCK-Km (40). The plasmid pKNOCKmZINT was electroporated into the TC142 strain, generating the TCZT14 strain (*troC* and *zinT* mutations). The plasmid pKNOCKmYCIC was electroporated into TC142, PS132 (25), PC135 (25), and SPP12 (25) to generate the double-mutant strains TCYC15 (*troC* and *yciC* mutations), ZAYC15 (*znuA* and *yciC* mutations), ZTYC15 (*zinT* and *yciC* mutations), and ZURYC15 (*zur* and *yciC* mutations), respectively.

Construction of plasmids expressing functional *troC*, *troCBA*, and *yciC* for complementation. DNA fragments of full-length *troC* (BT3809 [5′-ATGAACGATCCGTGCCTCAC-3′] and BT3810 [5′-AAATCAGCC ATGGGCATTCG-3′]; 837 bp), *troCBA* (BT3809 and BT4627 [5′-GAAA CTGTGCGTTTTACTGG-3′]; 2,747 bp), and *yciC* (BT5033 [5′-ATGAA AAAACTCCCTGTCAC-3′] and BT5034 [5′-AATCAGGCCGCCTGTC TATC-3′]; 1,205 bp) were amplified by PCR using *Pfu* DNA polymerase (Fermentas) and the specific primers. The PCR products were cloned into SmaI-digested pBBR1MCS-4 (41), generating pTROC, pTROCBA, and pYCIC, respectively.

RT-PCR. Total RNA was extracted from cells grown in LB at 28°C for 4 h and then treated with 1 mM EDTA for 15 min using a modified hot-phenol method (49). Reverse transcription (RT)-PCR was performed as previously described (49). Primer sets were used for amplifying the junction of *troC-troB* (BT4265 [5′-AGGCGCGTCATTTCCACGAG-3′] and BT4266 [5′-TGAGGCTCATGCGCCGCAAC-3′]; 280 bp) and *troB-troA* (BT4267 [5′-GCATGGTCTCCTGCTTTGCC-3′] and BT4268 [5′-CGATGATCGAGAAGCTGGCC-3′]; 310 bp). The PCR products were visualized using gel electrophoresis on a 1.8% agarose gel with ethidium bromide staining.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Agrobacterium tumefaciens		
NTL4	WT strain: a Ti plasmid-cured derivative of strain C58	37
PC135	*zinT*::pKNOCK-Gm (*zinT*::Gm); Gm^r	25
PS132	*znuA*::pKNOCK-Gm (*znuA*::Gm); Gm^r	25
SPP12	*zur*::pKNOCK-Gm (*zur*::Gm); Gm^r	25
TC142	*troC*::pKNOCK-Gm (*troC*::Gm); Gm^r	This study
TCZA15	*troC*::pKNOCK-Gm and *znuA*::pKNOCK-Km (*troC*::Gm *znuA*::Km); Gm^r and Km^r	This study
TCZT14	*troC*::pKNOCK-Gm and *zinT*::pKNOCK-Km (*troC*::Gm *zinT*::Km); Gm^r Km^r	This study
TCYC15	*troC*::pKNOCK-Gm and *yciC*::pKNOCK-Km (*troC*::Gm *yciC*::Km); Gm^r Km^r	This study
YC154	*yciC*::pKNOCK-Km (*yciC*::Km); Km^r	This study
ZAYC15	*znuA*::pKNOCK-Gm and *yciC*::pKNOCK-Km (*znuA*::Gm *yciC*::Km); Gm^r Km^r	This study
ZTYC15	*zinT*::pKNOCK-Gm and *yciC*::pKNOCK-Km (*zinT*::Gm *yciC*::Km); Gm^r Km^r	This study
ZURYC15	*zur*::pKNOCK-Gm and *yciC*::pKNOCK-Km (*zur*::Gm *yciC*::Km); Gm^r Km^r	This study
Escherichia coli		
DH5α	Host for general DNA cloning	38
BW20767	Host for plasmid pKNOCK-Gm and pKNOCK-Km	39
Plasmids for gene inactivation		
pKNOCK-Gm	Suicide vector; Gm^r	40
pKNOCK-Km	Suicide vector; Km^r	40
pKNOCKTROC	Internal coding region of *troC* cloned into pKNOCK-Gm; Gm^r	This study
pKNOCKmYCIC	Internal coding region of *yciC* cloned into pKNOCK-Km; Km^r	This study
pKNOCKmZINT	Internal coding region of *zinT* cloned into pKNOCK-Km; Km^r	This study
pKNOCKmZNUA	Internal coding region of *znuA* cloned into pKNOCK-Km; Km^r	25
Plasmids for complementation		
pBBR1MCS-4	Expression vector; Ap^r (pBBR)	41
pTROC	Full-length *troB* cloned into pBBR1MCS-4; Ap^r	This study
pTROCBA	Full-length *troCBA* operon cloned into pBBR1MCS-4; Ap^r	This study
pYCIC	Full-length *yciC* cloned into pBBR1MCS-4; Ap^r	This study
pZINT	Full-length *zinT* cloned into pBBR1MCS-4; Ap^r	25
pZNUA	Full-length *znuA* cloned into pBBR1MCS-4; Ap^r	25
pZUR	Full-length *zur* cloned into pBBR1MCS-4; Ap^r	25
Promoter-*lacZ* transcriptional fusions		
pUFR027lacZ	Promoter probe vector; Tc^r	42
p027troC-lacZ	266-bp DNA fragment containing *troC* promoter fused to *lacZ* of pUFR027lacZ	This study
p027yciC-lacZ	274-bp DNA fragment containing *yciC* promoter fused to *lacZ* of pUFR027lacZ	This study
YciC-LacZ and YciC-PhoA translational fusions		
pPR9TT	Reporter vector, LacZ as a reporter; Ap^r	43
pYCLacZ	YciC amino acid residues 1–400 fused to LacZ of pPR9TT; Ap^r	This study
p'PhoA	5′-truncated *phoA* gene lacking its signal peptide sequence cloned into pBBR; Ap^r	44
pYCPhoA	YciC amino acid residues 1–400 fused to PhoA of p′PhoA; Ap^r	This study
Plasmid for tumor assay		
pCMA1	pTiC58*traM*::*nptII*; Km^r	45

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

qRT-PCR analysis. Quantitative real-time (qRT)-PCR was performed as previously described (50). Log-phase cells grown in LB were not treated or were treated with metal and a metal chelator for 15 min prior to harvest. The metals $CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$ were used at a final concentration of 0.45 mM. The metal chelator used was EDTA (1 mM). Gene-specific primers for *troC* (BT3751 [5′-TGAAACCGCTCGGCGGTGAG-3′] and BT3752 [5′-CTGCGCATC CTGCAACATGG-3′]; 293 bp), *yciC* (BT5027 [5′-GTGATGGCGACAA CCGCACC-3′] and BT5028 [5′-GCCCTGGCGAAATCGAAGCG-3′]; 223 bp), and the 16S rRNA gene (BT1421 [5′-GAATCTACCCATCTCT GCGG-3′] and BT1422 [5′-AAGGCCTTCATCACTCACGC-3′]; 280 bp) were used.

The amount of a specific mRNA target was normalized to the amount of a housekeeping gene 16S rRNA. Fold changes in gene expression are relative to untreated samples from wild-type NTL4 using the $2^{-\Delta\Delta Ct}$ method (51). The data are reported as the means of biological triplicates and standard deviations (SD).

Determination of the transcriptional start site for *troC* and *yciC* using 5′ RACE. RNA samples were isolated from wild-type NTL4 log-phase cells grown in LB and treated with 1 mM EDTA for 15 min. 5′ Rapid

amplification cDNA ends (RACE) (Roche) was performed according to the manufacturer's instructions. The specific primers SP1 and SP2 for *troC* are BT3752 (5′-CTGCGCATCCTGCAACATGG-3′) and BT3758 (5′-TAGCGCCATCCAGATGATGC-3′), respectively. The specific primers SP1 and SP2 for *yciC* are BT5028 (5′-GCCCTGGCGAAATCGAAGCG-3′) and BT5042 (5′-CGAGGATGTGGTTGAGAAGG-3′), respectively.

Construction of promoter-*lacZ* transcriptional fusions. DNA fragments containing the promoter region of *troC* (BT3757 [5′-TATGTGCGACATGTCAACGG-3′] and BT3758 [5′-TAGCGCCATCCAGATGATGC-3′]; 269 bp) and *yciC* (BT5041 [5′-TTAGAGTGGCGCGCTGTTGG-3′] and BT5042 [5′-CGAGGATGTGGTTGAGAAGG-3′]; 274 bp) were amplified from *A. tumefaciens* NTL4 genomic DNA using PCR. The PCR products were cloned into a unique HindIII site (and end-gap filled with Klenow enzyme) of the promoter probe vector pUFR027lacZ, a derivative of pUFR027 (42), to generate the plasmids p027troC-lacZ and p027yciC-lacZ, respectively.

β-Galactosidase activity assay. β-Galactosidase (β-Gal) activity was measured as described by Miller (52). Log-phase cells grown in LB were not treated or were treated with 1 mM EDTA for 1 h. The cells were then harvested. Crude bacterial cell lysates were prepared as previously described (48). Protein concentrations were determined using the Bradford Bio-Rad protein assay. Specific activity was calculated in units per milligram of protein. Data are reported as the means of biological triplicates ± SD.

EDTA sensitivity test. Log-phase cells grown in LB were adjusted, serially diluted, and spotted onto plates containing AB medium (47) and AB plus EDTA (0.3, 0.6, 0.9, 1, 1.1, 1.2, 1.3, 1.4, and 1.5 mM) according to a protocol previously described (25). In some experiments, AB plates containing 1.2 mM EDTA were individually supplemented with 50 μM $ZnCl_2$, $CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MgCl_2$, $MnCl_2$, or $NiCl_2$. The plates were then incubated at 28°C for 48 h. Each strain was tested in duplicate, and the experiment was repeated at least twice.

Construction of *yciC-lacZ* and *yciC-phoA* translational fusions. Primers BT5041 (5′-TTAGAGTGGCGCGCTGTTGG-3′) and BT5309 (5′-GCCGCCTGTCTATCCCAGT-3′) were used to amplify the *yciC* promoter region and sequences encoding the entire 400 amino acids of YciC. The DNA fragments were cloned into the SmaI sites of the plasmid vectors pPR9TT (43) and p′PhoA (44) to generate plasmids pYCLacZ and pYCPhoA, respectively. The β-galactosidase and alkaline phosphatase activities were assayed as previously described (44), using wild-type NTL4 carrying plasmids pYCLacZ and pYCPhoA, which express the hybrid proteins YciC-LacZ and YciC-PhoA, respectively.

Measurement of total cellular zinc content. Zinc ions were measured in parts per billion using an inductively coupled plasma mass spectrometer (ICP-MS), as previously described (44). Samples were prepared from cells grown in LB plus 0.5 mM EDTA at 28°C for 24 h. The data are reported as the means of biological triplicates and SD.

Virulence assay. *A. tumefaciens* strains carrying plasmid pCMA1 were used to infect young *Nicotiana benthamiana* plants as previously described (25). Cells grown in LB plus 0.5 mM EDTA at 28°C for 24 h were washed and resuspended in induction broth (47), pH 5.5 (IB 5.5), plus 300 μM acetosyringone (AS). The cells were incubated at 28°C with shaking for 20 min, harvested, and adjusted to an optical density at 600 nm (OD_{600}) of 0.1 in IB 5.5 plus 300 μM AS. A 5-μl aliquot of the cell suspension was inoculated into a wounded *N. benthamiana* petiole. Each bacterial strain was used to infect 15 petioles. Tumor formation at 4 weeks after infection was assessed.

RESULTS

The *A. tumefaciens troCBA* operon and *yciC* are negatively regulated by Zur. *A. tumefaciens* genome sequence analysis (53) revealed that the gene arrangement in the *tro* operon starts with *troC* (Atu3180), followed by *troB* (Atu3179) and *troA* (Atu3178) (Fig. 1A), and these genes may be cotranscribed. RT-PCR analysis was performed, confirming that *troC*, *troB*, and *troA* are cotranscribed (see Fig. S1 in the supplemental material). The *A. tumefaciens troCBA* operon and *yciC* are divergently oriented. The translational start codons of *troC* and *yciC* are separated by 300 bp (Fig. 1B). The transcriptional start sites of *troC* (at the A residue) and of *yciC* (at the A residue) were determined by 5′ RACE, and the −10 and −35 sequences were predicted using BPROM (Softberry) (Fig. 1B). A Zur-binding site (5′-TTAATGTTATTTCATTAC-3′; underlined nucleotides are the center of symmetry for the inverted palindrome) was identified previously in the intergenic region between *A. tumefaciens troCBA* and *yciC* (26) and overlaps the predicted −35 site of *troC* (Fig. 1B). Furthermore, we identified another Zur-binding site (5′-GTAATGTTATTACGTTAC-3′) that overlaps the predicted −10 site of *yciC* (Fig. 1B). An alignment of *A. tumefaciens* Zur boxes found in the Zur-regulated genes is shown in Fig. 1C.

To assess the promoter activity of *troC* and *yciC*, the DNA fragments upstream of the translational start codon, predicted −10 and −35 sequences, and a potential Zur-binding site for the *troC* promoter or for the *yciC* promoter were fused to a promoterless *lacZ* reporter gene (transcriptional fusions) (plasmids p027troC-lacZ and p027yciC-lacZ, respectively) (Fig. 1D), and β-Gal activity was measured. In wild-type NTL4 (the WT), β-Gal activities from both *troC-lacZ* and *yciC-lacZ* fusions were increased when cells were treated with EDTA (WT/pBBR) (Fig. 1E). β-Gal in the *zur* mutant strain, SPP12, was constitutively expressed at high levels under all the tested conditions (*zur*::Gm/pBBR) (Fig. 1E); however, expression of the *zur* gene from a multicopy plasmid, pZUR, could suppress this phenotype (*zur*::Gm/pZUR) (Fig. 1E).

The *A. tumefaciens troCBA* operon and *yciC* are inducible with zinc depletion. Expression of the *troC* and *yciC* genes was determined using qRT-PCR. As expected for genes involved in metal acquisition, expression of the *troC* and *yciC* genes in the WT was inducible (~10^3-fold) by the metal chelator EDTA compared to untreated cells. Both genes were constitutively expressed at high levels in the *zur* mutant but were suppressed when complemented with pZUR (Fig. 2A). These results further supported the view that *A. tumefaciens* Zur negatively regulates *troCBA* and *yciC*. To investigate the metal-specific responses of *troC* and *yciC*, repression of the EDTA-induced expression by various metals was determined. In the wild type, Zn^{2+} was the most potent metal ion that could repress the EDTA-induced expression of *troC* and *yciC* (Fig. 2B) compared to other metals, including cadmium, cobalt, copper, iron, magnesium, manganese, and nickel. These results demonstrated that the *A. tumefaciens troCBA* operon and *yciC* are inducible, specifically by zinc depletion, which supports the view that *A. tumefaciens* TroCBA and YciC are involved in zinc acquisition.

The *troC* mutant is hypersensitive to EDTA treatment, and inactivation of *yciC* further increases the sensitivity; however, the TroCBA transporter can function independently of YciC. *A. tumefaciens* mutant strains were constructed to assess the physiological functions of *troC* (TC142) and *yciC* (YC154). Growth was determined under metal limitation in the presence of EDTA (1, 1.1, 1.2, 1.3, 1.4, and 1.5 mM [see Fig. S2 in the supplemental material]). Inactivation of *troC*, but not *yciC*, caused cells to become hypersensitive to EDTA. Consistent with a previous report (25), the *znuA* mutant (PS132) showed no phenotype, while the *zinT* mutant (PC135) was hypersensitive to EDTA (Fig. 3A). Similar to the *zinT* mutant, the *troC* mutant was ~10^2-fold more

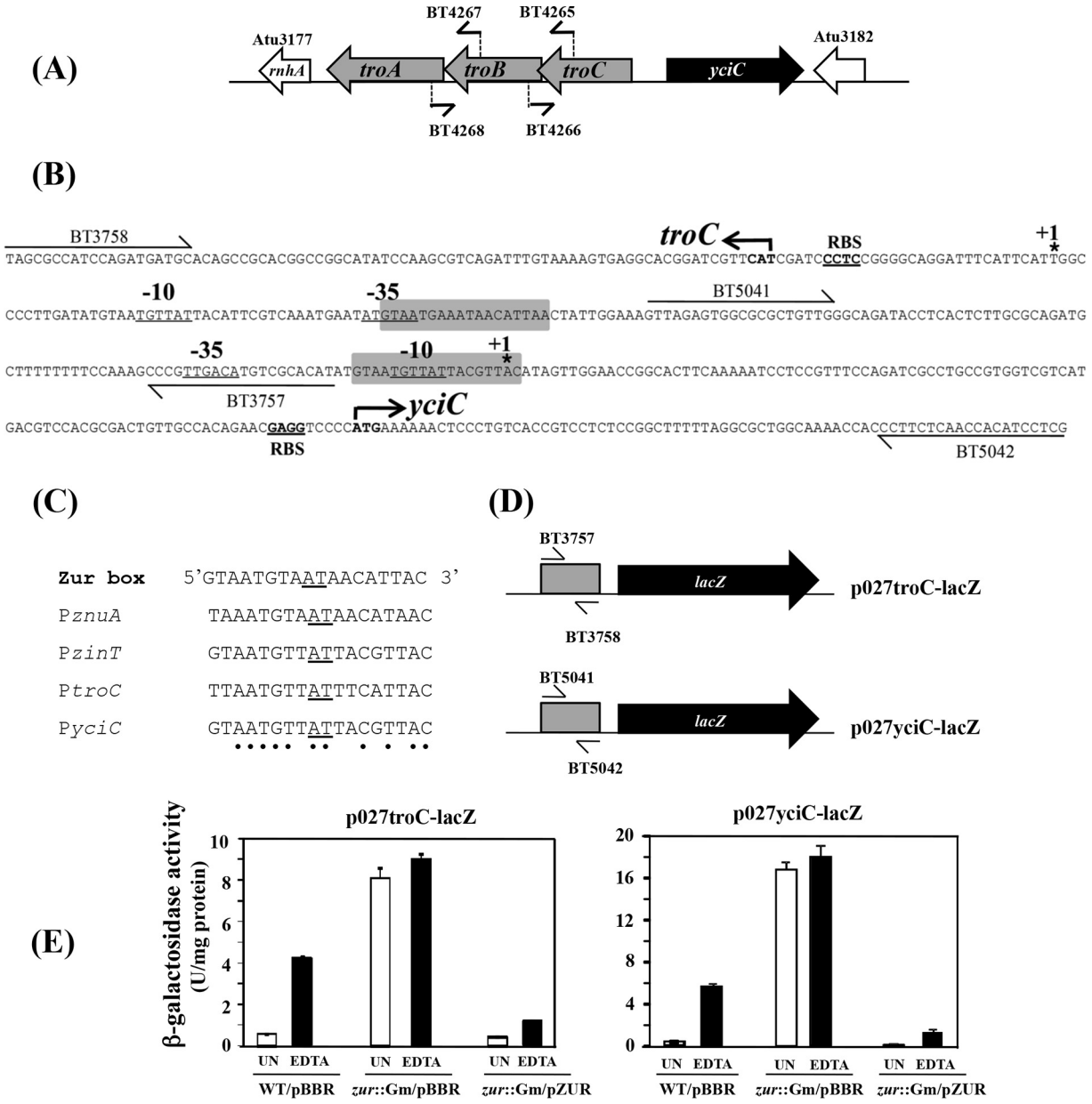

FIG 1 (A) Genomic context of the *A. tumefaciens troCBA* operon and *yciC*. The *rnhA* (Atu3177) gene encoding RNase H is located downstream of the *troCBA* operon. The Atu3182 gene encoding a hypothetical protein with unknown function is located downstream of the *yciC* gene. The primer sets used to amplify the junctions between *troC* and *troB* (BT4265 and BT4266) and between *troB* and *troA* (BT4267 and BT4268) with RT-PCR analysis are indicated. (B) *troC* and *yciC* promoters. The ATG start codons for *troC* and *yciC* are shown in boldface with bent arrows. The transcriptional +1 start site of *troC* and *yciC* was determined using 5′ RACE and is indicated by asterisks. The predicted −10 and −35 sequences are underlined. The Zur-binding sites are shaded. The putative ribosome-binding sites (RBS) are underlined. (C) Conserved Zur-binding site (Zur box) for *A. tumefaciens* (14). The sequences of Zur boxes found in the promoter regions of *A. tumefaciens znuA*, *zinT*, *troC*, and *yciC* (P*znuA*, P*zinT*, P*troC*, and P*yciC*, respectively) are shown. The conserved residues in all the Zur boxes are marked with dots. (D) Schematic representation of the promoter-*lacZ* fusions (not drawn to scale) from plasmids p027troC-lacZ and p027yciC-lacZ. (E) β-Galactosidase activity assay. Wild-type NTL4 (WT/pBBR), the *zur* mutant strain SPP12 (*zur*::Gm/pBBR), and the complemented strain (*zur*::Gm/pZUR) contain either p027troC-lacZ or p027yciC-lacZ. pBBR is a plasmid vector, while pZur contains a functional *zur* gene. Cells were grown in LB for 4 h and then were left untreated (UN) or treated with 1 mM EDTA for 1 h. The results are the means and SD of triplicate samples.

sensitive to 1.2 mM EDTA than the WT (Fig. 3A) and could be fully rescued by the addition of 50 μM $ZnCl_2$ (Fig. 3A, 1.2 mM EDTA plus Zn) but not by other metals (see Fig. S3A in the supplemental material).

The EDTA-sensitive phenotype of the *troC* mutant (TC142) could not be reversed by the plasmid pTROC (see Fig. S3B in the supplemental material), suggesting that the *troC* gene knockout by insertional inactivation used to generate the TC142 strain may have a polar effect on other downstream genes in the *troCBA* operon. This notion was supported by the observation that the plasmid pTROCBA could fully restore the growth of the *troC* mutant (Fig. 3B; see Fig. S3B in the supplemental material).

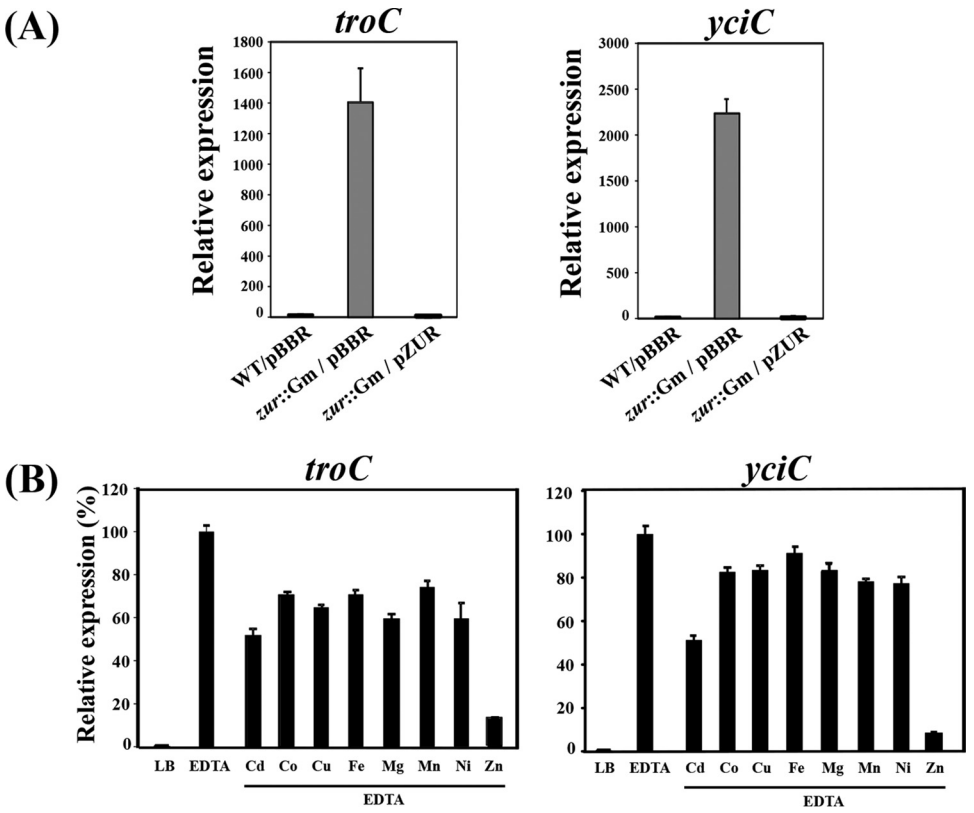

FIG 2 (A) Expression analysis of *troC* and *yciC* using qRT-PCR. (A) RNA samples were isolated from log-phase cells of the wild type (WT/pBBR), the *zur* mutant (*zur*::Gm/pBBR), and the complemented strain (*zur*::Gm/pZUR) grown in LB medium. pBBR is the plasmid vector. pZUR is the plasmid containing a functional *zur* gene. The expression of the target genes was normalized to that of 16S rRNA, and the fold changes in gene expression in *zur*::Gm/pBBR and *zur*::Gm/pZUR are relative to those in WT/pBBR (regarded as 1). The experiment was performed in biological triplicate, and the error bars indicate the standard deviations. (B) Induction of *troC* and *yciC* is specific to zinc limitation. Shown is qRT-PCR analysis of *troC* and *yciC* expression. Wild-type NTL4 cells were grown in LB medium and under metal-limiting conditions (LB plus 1 mM EDTA). Metals ($CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$) were supplemented at a final concentration of 0.45 mM. The expression levels are presented as percentages and are relative to those in cells grown in LB plus 1 mM EDTA (100%).

Although the *yciC* mutant strain showed no phenotype (Fig. 3A; see Fig. S2 and S4 in the supplemental material), the inactivation of *yciC* further increased the sensitivity of the *troC* mutant to EDTA (Fig. 3B). Growth of the *troC* mutant strain TC142 (*troC*::Gm/pBBR) on the AB plate containing 0.6 mM EDTA was similar to that of the WT (WT/pBBR), while the TCYC15 strain lacking both *troC* and *yciC* (*troC*::Gm *yciC*::Km/pBBR) was ~10^3-fold more sensitive to 0.6 mM EDTA than the WT (Fig. 3B). Expressing the functional *yciC* gene from the plasmid pYCIC could restore the growth defect of TCYC15 (*troC* and *yciC* mutations) to levels similar to that of TC142 (*troC* mutation) and the WT at 0.6 mM EDTA, but not at higher levels of EDTA (0.9 and 1.2 mM) (*troC*::Gm *yciC*::Km/pYCIC) (Fig. 3B). In contrast, at 0.6, 0.9, and 1.2 mM EDTA, the EDTA-hypersensitive phenotype of TCYC15 could be fully reversed to WT by complementation with pTROCBA (*troC*::Gm *yciC*::Km/pTROCBA) (Fig. 3B), demonstrating that *A. tumefaciens* requires the TroCBA transporter to survive under severe metal-limiting conditions and that TroCBA can function even in the absence of YciC.

The important role of ZnuA is revealed when TroCBA is disrupted. Inactivation of either *znuA* or *znuAB* had no detectable effect on the growth of *A. tumefaciens* on the AB plate containing 1.4 mM EDTA compared to the WT (see Fig. S2 in the supplemental material) (25). However, disruption of *znuA* in combination with *troC* (TCZA15) led to a growth defect of *A. tumefaciens* in a minimal AB medium and hypersensitivity to EDTA (*troC*::Gm *znuA*::Km) (Fig. 4). The growth defect of TCZA15 could be rescued by zinc supplementation (1.2 mM EDTA plus Zn) (Fig. 4). Furthermore, the EDTA sensitivity of TCZA15 (*troC* and *znuA* mutations) could be reversed to that of TC142 (*troC* mutation) by complementation with the plasmid pZNUA (*troC*::Gm *znuA*::Km/pZNUA) (Fig. 5A), but not by the plasmid pZINT or pTROA. Although TCZA15 showed a growth defect on the AB plate (a minimal medium), it could grow on the LA plate (a rich medium) similarly to the WT (*troC*::Gm *znuA*::Km/pBBR) (Fig. 5A). These results demonstrated the role of *A. tumefaciens* ZnuA (a periplasmic zinc-binding protein) in zinc acquisition under zinc limitation and that *A. tumefaciens* ZnuA function could not be substituted for by other periplasmic zinc-binding proteins, such as ZinT and TroA.

ZinT may interact with TroCBA. It has been reported previously that *A. tumefaciens* ZinT functions independently of ZnuABC (25). The inactivation of *zinT* caused a reduction in the total zinc content and hypersensitivity to EDTA (25). To test the possibility of interaction between *A. tumefaciens* ZinT (a periplasmic protein) and TroCBA in zinc transport, a strain (TCZT14) containing mutations in *troC* and *zinT* was generated, and its sensitivity to EDTA was determined. The TC142 (*troC* mutation) and

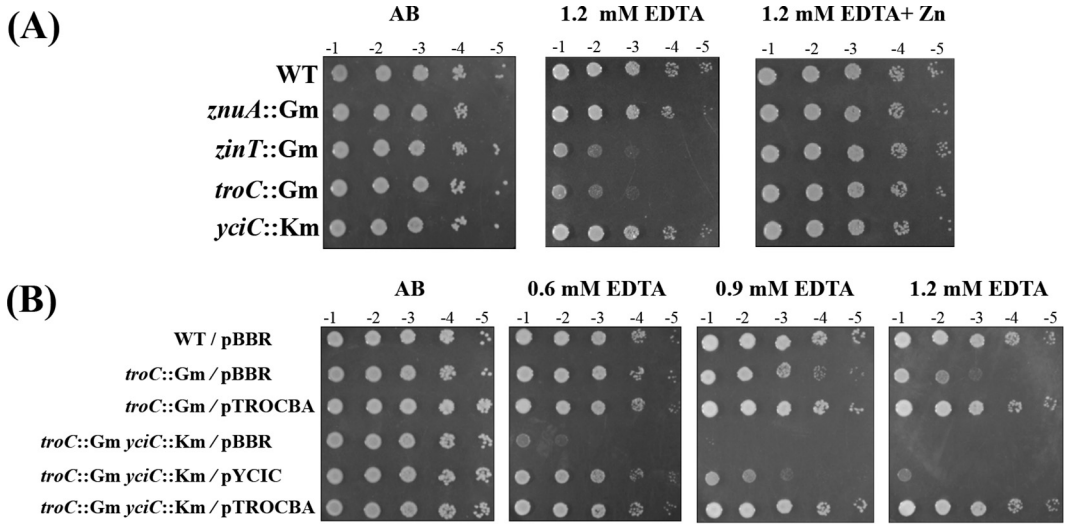

FIG 3 Effects of a single mutation (A) and double mutations (B) in the *troC* and *yciC* genes on the sensitivity of *A. tumefaciens* to EDTA. (A) The strains are wild-type NTL4 (WT), PS132 (*znuA*::Gm), PC135 (*zinT*::Gm), TC142 (*troC*::Gm), and YC154 (*yciC*::Km). (B) The WT, TC142 (*troC*::Gm), and TCYC15 (*troC*::Gm *yciC*::Km) strains carry the plasmid vector (pBBR). The mutant strains were complemented with functional *troCBA* or *yciC* from the multicopy plasmids pTROCBA and pYciC, respectively. Cells were adjusted, serially diluted, and spotted onto plates containing AB and AB plus EDTA (0.6, 0.9, and 1.2 mM) with or without 50 μM $ZnCl_2$. Tenfold serial dilutions are indicated. The plates were incubated at 28°C for 48 h.

TCZT14 (*troC* and *zinT* mutations) strains were ~10^2-fold and ~10^3-fold, respectively, more sensitive to 1.2 mM EDTA than the WT (Fig. 4). Moreover, the EDTA-hypersensitive phenotype of TCZT14 (*troC* and *zinT* mutations) could not be reversed to that of TC142 (*troC* mutation) by complementation with the plasmid pZINT (Fig. 5B), but the EDTA-hypersensitive phenotype of PC135 (*zinT* mutation) could be fully restored by pZINT (Fig. 5B). The results suggested that TroCBA is required for the function of *A. tumefaciens* ZinT.

Loss of the zinc chaperones ZinT and YciC causes a severe EDTA-mediated growth defect. Double mutations in *zinT* and *yciC* (ZTYC15) led to hypersensitivity to EDTA, and this EDTA-dependent growth defect could be detected at 0.3 mM EDTA (~10^3-fold more sensitive than the wild type) (Fig. 4). At 0.3 and 0.6 mM EDTA, the growth defect of ZTYC15 could be fully restored to PC135 (*zinT* mutation) by complementation with the plasmid pZINT or pYCIC (Fig. 5C). At 1.2 mM EDTA, complementation with either pZINT or pYCIC did not rescue ZTYC15 (Fig. 5C), but addition of zinc did (1.2 mM EDTA plus Zn) (Fig. 4). These results demonstrated that *A. tumefaciens* requires both zinc chaperones, ZinT and YciC, to cope with severe zinc-limiting conditions.

$YciC_{At}$ is a cytoplasmic protein. *B. subtilis* YciC ($YciC_{Bs}$) is a membrane protein (27). Unlike $YciC_{Bs}$, *A. tumefaciens* YciC ($YciC_{At}$) is likely to reside in the cytoplasm. The hydropathy of $YciC_{At}$ was determined using the Kyte-Doolittle hydropathy plot (54). The hydropathy plot and other programs, including HMMTOP (55) and Phobius (56), predicted that there would be no transmembrane segment in the $YciC_{At}$ protein. To determine the localization of $YciC_{At}$, the entire 400 amino acids of $YciC_{At}$ was fused to PhoA (alkaline phosphatase) and LacZ (β-galactosidase) to generate $YciC_{At}$-PhoA and $YciC_{At}$-LacZ fusions using a previously described protocol (44). The alkaline phosphatase and β-galactosidase activities of the hybrid proteins could be used as indicators for the periplasmic and cytoplasmic locations, respectively, of the target protein fusion sites (57, 58). The $YciC_{At}$-PhoA fusion displayed low levels of alkaline phosphatase activity (~0.22 U), while the $YciC_{At}$-LacZ fusion exhibited high levels of β-galactosidase activity (~240 U) compared to the negative-control vectors, p′PhoA (~0.04 U) and pPR9TT (~8 U), which showed low ac-

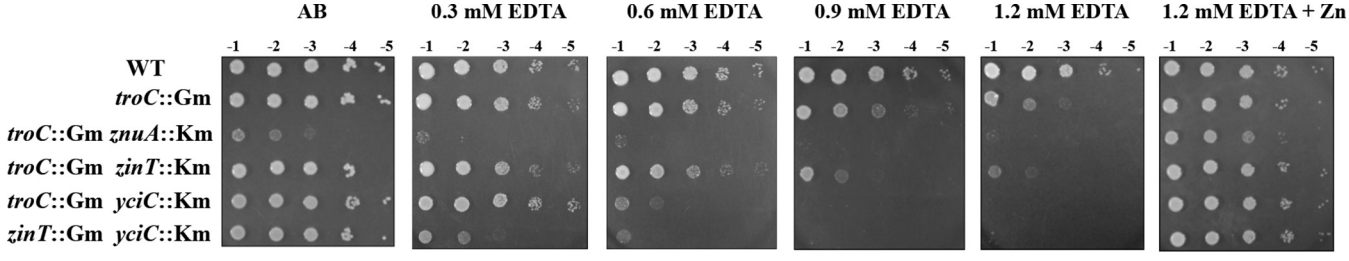

FIG 4 EDTA-sensitive phenotypes of strains containing double mutations of genes encoding zinc uptake proteins and zinc chaperones. The EDTA sensitivities of TCZA15 (*troC*::Gm *znuA*::Km), TCZT14 (*troC*::Gm *zinT*::Km), TCYC15 (*troC*::Gm *yciC*::Km), and ZTYC15 (*zinT*::Gm *yciC*::Km) were compared to that of wild-type NTL4 (WT) and TC142 (*troC*::Gm). Sensitivity to EDTA on plates containing AB and AB plus EDTA (0.3, 0.6, 0.9, and 1.2 mM) with or without 50 μM $ZnCl_2$ was assessed as described for Fig. 3.

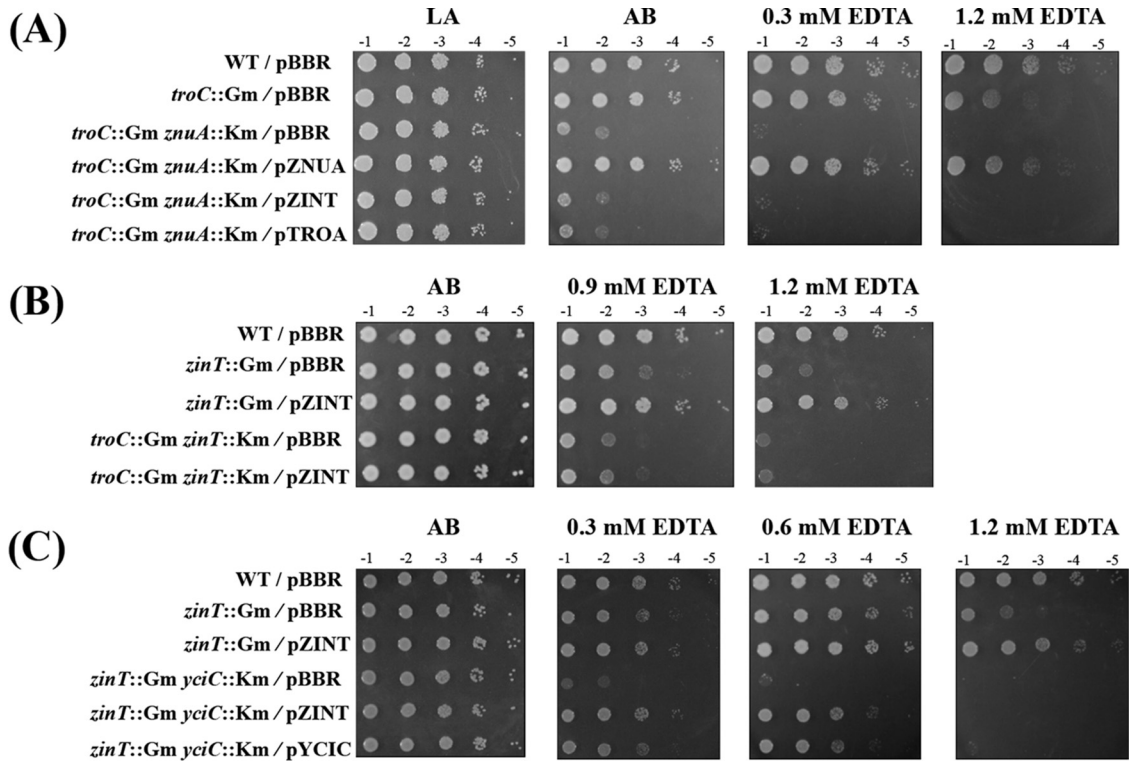

FIG 5 (A) ZnuA under metal limitation conditions could not be replaced by the periplasmic zinc-binding proteins ZinT and TroA. The TCZA15 (*troC*::Gm *znuA*::Km) strain was complemented with functional *znuA*, *zinT*, or *troA* from the plasmids pZNUA, pZINT, and pTROA, respectively. The cells were tested on plates containing LA (rich medium) and AB (minimal medium). Sensitivity to EDTA on AB plates containing EDTA (0.3, 0.6, 0.9, and 1.2 mM) was assessed as described for Fig. 3. (B) ZinT could not function when TroCBA was inactivated. The PC135 (*zinT*::Gm) and TCZT14 (*troC*::Gm *zinT*::Km) strains were complemented with functional *zinT* from the plasmid pZINT. (C) Important role of zinc chaperones ZinT and YciC. The ZTYC15 (*zinT*::Gm *yciC*::Km) strain was complemented with functional *zinT* or *yciC* from the plasmids pZINT and pYCIC, respectively.

tivity of alkaline phosphatase and β-galactosidase, respectively. The results suggest that the $YciC_{At}$ protein is a cytoplasmic protein.

$YciC_{At}$ may possibly catalyze GTP hydrolysis to drive the $TroCBA_{At}$ transporter. However, $YciC_{At}$ is not essential for the $TroCBA_{At}$ transporter (Fig. 3B). Another possible function of $YciC_{At}$ is as a zinc chaperone that may transfer zinc ions to the zinc sensor Zur_{At}. To test this idea, expression of the *znuA*, *zinT*, and *troC* genes in the *yciC*::Km strain was determined by qRT-PCR. Similarly to the WT, EDTA induction and zinc repression of *znuA*, *zinT*, and *troC* were observed in the *yciC*::Km strain (data not shown). Therefore, $YciC_{At}$ is unlikely to function in zinc loading to Zur_{At} for mediating repression of the Zur_{At} regulon.

Decreased zinc levels in the EDTA-hypersensitive mutants. Total zinc content in the mutant strains compared to the WT was determined using ICP-MS (Fig. 6A). Because the *troC znuA* double-mutant strain has a growth defect in the minimal AB medium (Fig. 4), ICP-MS was performed using cells grown in LB (rich medium) for 24 h; however, the zinc levels in the mutant strains were not dramatically different from those in the WT (data not shown). When cells were grown in LB plus 0.5 mM EDTA for 24 h, all the EDTA-hypersensitive mutants showed an obvious reduction in zinc content compared to the WT (Fig. 6A). It should be noted that 0.5 mM EDTA in LB did not inhibit the growth of the WT and all the tested mutant strains (data not shown). The lowest levels of intracellular zinc were detected in the *troC*::Gm *znuA*::Km strain (*troC*::Gm *yciC*::Km and *troC*::Gm *zinT*::Km strains < *troC*::Gm and *zinT*::Gm *yciC*::Km strains < *zinT*::Gm strain) (Fig. 6A). The WT and the *yciC*::Km and *znuA*::Gm *yciC*::Km mutant strains showed similar levels of EDTA sensitivity (see Fig. S4 in the supplemental material) and intracellular zinc levels (Fig. 6A).

Virulence assay. *N. benthamiana* plants were infected with the WT and the mutant strains carrying pCMA1 (a tumor-inducing plasmid). The mutant strains TC142 (*troC* mutation), TCYC15 (*troC* and *yciC* mutations), TCZT14 (*troC* and *zinT* mutations), and ZTYC15 (*zinT* and *yciC* mutations) were tested. All *A. tumefaciens* strains grown in LB for 24 h caused similar tumor formation on infected plants (data not shown). Next, the *A. tumefaciens* strains were grown under metal limitation in LB plus 0.5 mM EDTA for 24 h and used to infect plants. However, TC142, TCYC15, TCZT14, and ZTYC15 all caused tumors on the infected plants and were similar to the WT (Fig. 6B). Unfortunately, several attempts were made, but we could not select the TCZA15 (*troC* and *znuA* mutations) strain carrying pCMA1; thus, we were unable to determine the virulence of TCZA15.

DISCUSSION

The high-affinity zinc importer ZnuABC is found widely in many bacteria. Rarely, some bacteria have a second high-affinity zinc uptake system in addition to ZnuABC, such as *T. pallidum* TroABCD ($TroABCD_{Tp}$) (29, 30) and *Haemophilus influenzae*

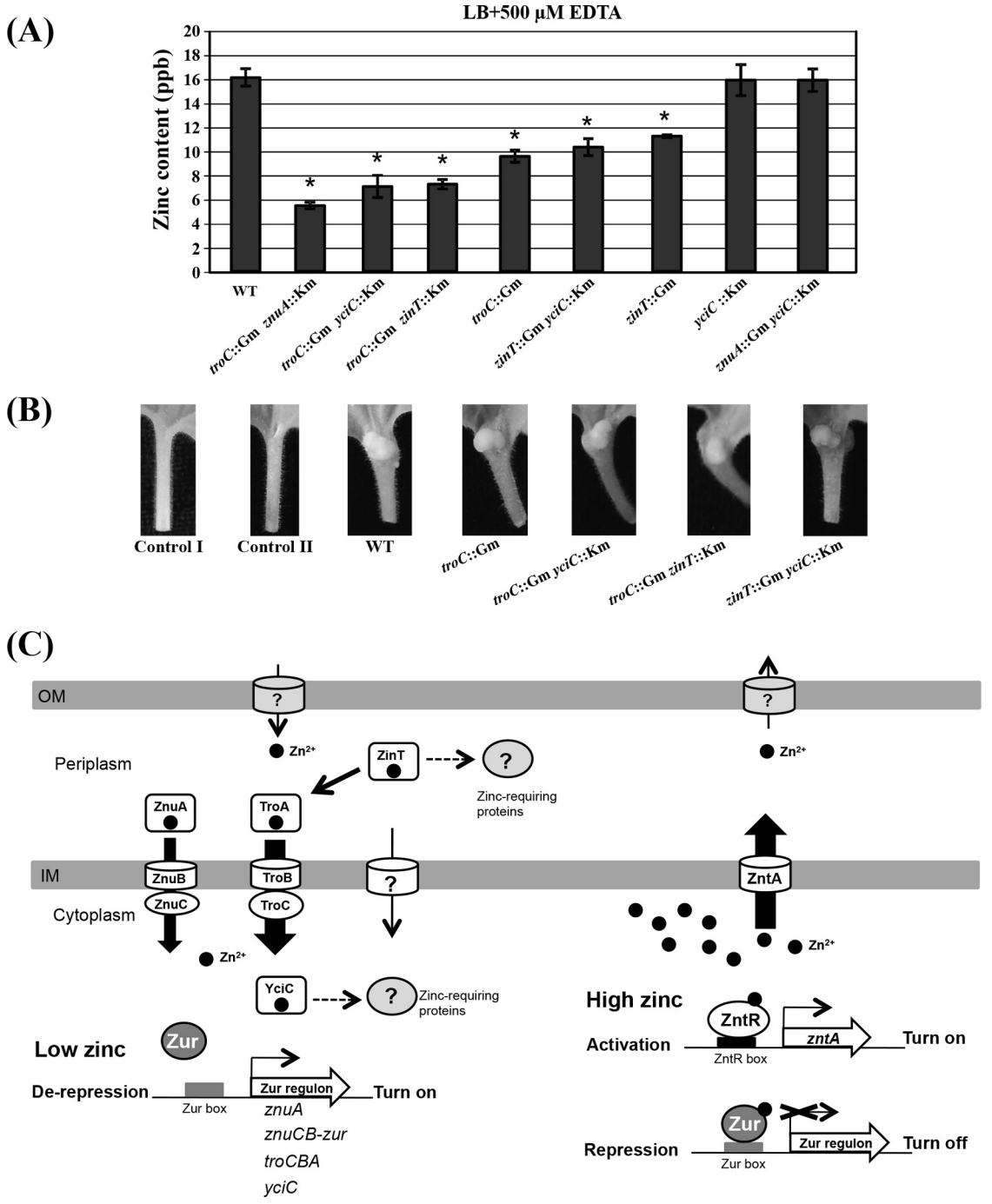

FIG 6 (A) Intracellular zinc content was determined using ICP-MS. The WT and the mutant strains TCZA15 (*troC*::Gm *znuA*::Km), TCYC15 (*troC*::Gm *yciC*::Km), TCZT15 (*troC*::Gm *zinT*::Km), TC142 (*troC*::Gm), ZTYC15 (*zinT*::Gm *yciC*::Km), PC135 (*zinT*::Gm), YC154 (*yciC*::Km), and ZAYC15 (*znuA*::Gm *yciC*::Km) were grown in LB plus 0.5 mM EDTA for 24 h. The results are shown as the means of samples in triplicate, and the error bars indicate the standard deviations. The bars marked with asterisks are significantly different from the WT ($P < 0.05$ in an unpaired Student's *t* test). (B) Virulence assay. Shown is tumor formation on the petioles of *N. benthamiana* plants infected with the WT and the mutant strains TC142 (*troC*::Gm), TCYC15 (*troC*::Gm *yciC*::Km), TCZT15 (*troC*::Gm *zinT*::Km), and ZTYC15 (*zinT*::Gm *yciC*::Km) grown in IB 5.5 medium containing 300 μM AS. Control I, without inoculation; control II, inoculation of the wounded petiole with IB 5.5 plus 300 μM AS. (C) Proposed model of zinc homeostasis. *A. tumefaciens* contains two zinc sensors, Zur and ZntR, which control zinc homeostasis. Zur is the transcriptional repressor of zinc acquisition genes, including *znuA*, *znuBC*, *zinT*, *troCBA*, and *yciC*. ZntR is a transcriptional activator of a zinc efflux gene, *zntA*. Under low-zinc conditions, Zur is in the apo form and dissociates from DNA to trigger expression of the Zur regulon. The TroABC system is the major zinc uptake system (thick solid arrow) compared to ZnuABC. ZinT functions in association with TroCBA. ZinT and YciC may function as zinc chaperones that supply Zn^{2+} (black dots) to zinc-requiring proteins in the periplasm and cytoplasm, respectively. When the intracellular zinc levels are high, the complexes ZntR-Zn^{2+} and Zur-Zn^{2+} are formed, which in turn activates zinc efflux by ZntA and represses zinc uptake systems, respectively. The outer membrane (OM) channels for zinc import and zinc extrusion have not been identified. Zinc ions may also be transported into the cytoplasm via unknown inner membrane (IM) transporters. ?, unknown; dashed arrows, possible mechanisms.

ZevAB (59). *Listeria monocytogenes* contains two zinc permease systems, ZurAM and ZinABC, which are regulated by Zur (60). The presence of two high-affinity zinc uptake systems helps these bacteria to survive in diverse zinc-limiting environments and during infection. In addition to ZnuABC, *A. tumefaciens* has a cluster of genes (Atu3178, Atu3179, and Atu3180) encoding an ABC-type transporter. The Atu3178 gene encodes a protein belonging to the TroA superfamily that has amino acid identity to $TroA_{Tp}$ (28%), ZinA (26%), and *A. tumefaciens* ZnuA (25%). Therefore, we named Atu3178, Atu3179, and Atu3180 *troA*, *troB*, and *troC*, respectively. In other bacteria, *tro* operons are involved in metal uptake and can transport either zinc or manganese and possibly iron (29, 33–36). The *A. tumefaciens troCBA* operon ($troCBA_{At}$) was shown here to respond specifically to zinc limitation.

To survive under a wide range of zinc-deficient conditions, *A. tumefaciens* requires the cooperation of Zur-regulated zinc acquisition systems, including two ABC-type zinc importers, $TroCBA_{At}$ and $ZnuABC_{At}$, and two zinc chaperones, $ZinT_{At}$ and $YciC_{At}$. Zinc uptake in *A. tumefaciens* differs from the systems in many other bacteria in that $TroCBA_{At}$ is the major zinc importer while $ZnuABC_{At}$ plays a lesser role. $ZnuABC_{At}$ is very important for supporting growth under zinc limitation in the absence of $TroCBA_{At}$. This indicates that $ZnuABC_{At}$ may function as a backup system for high-affinity zinc uptake. It is also possible that the two transporters function optimally under different conditions, such as different pHs. Consequently, $TroCBA_{At}$ may play a dominant role in zinc uptake under laboratory test conditions and $ZnuABC_{At}$ may play a more crucial role under other, as yet unidentified conditions. The severe growth defect of the *troC*::Gm *znuA*::Km strain could be rescued by supplementation with zinc, suggesting that zinc can be taken up through additional low-affinity zinc transporters.

ZinT proteins in *Escherichia coli* O157 (20) and *S. enterica* (22, 23) ($ZinT_{Ec}$ and $ZinT_{Se}$, respectively) are accessory components of the ZnuABC transporters that enhance the ability of cells to recruit zinc under severe zinc shortage. In contrast to $ZinT_{Ec}$ and $ZinT_{Se}$, $ZinT_{At}$ plays a critical role for survival under zinc depletion and can function in the absence of $ZnuABC_{At}$ (25). It was found that $ZinT_{At}$ may interact with $TroCBA_{At}$. It is likely that $ZinT_{At}$ is an additional component of the $TroCBA_{At}$ transporter, and it may help increase the rate of zinc delivery to $TroCBA_{At}$ when *A. tumefaciens* faces severe zinc deprivation. $ZinT_{At}$ may also supply zinc ions to other periplasmic zinc-requiring proteins.

The members of the COG0523 subfamily of G3E-GTPases have been reported to function as metal insertases by using GTP hydrolysis to drive the incorporation of a metal cofactor into the catalytic site of the target enzyme and/or as metal chaperones to allocate a metal cofactor to the target zinc-requiring proteins (26). *B. subtilis* YciC ($YciC_{Bs}$) belongs to a subgroup, Zur-regulated COG0523 proteins. $YciC_{Bs}$ is a membrane protein, and it was proposed to be a component of a low-affinity zinc transporter and possibly a zinc chaperone (27, 28). Unlike $YciC_{Bs}$, $YciC_{At}$ is a cytoplasmic protein. Inactivation of $yciC_{At}$ in combination with the mutation of a gene encoding the major zinc importer, $troC_{At}$, or the periplasmic zinc chaperone, $zinT_{At}$, caused a growth defect on LA plates containing EDTA. When *A. tumefaciens* encounters conditions of severe zinc shortage, even if the high-affinity zinc uptake $TroCBA_{At}$ and $ZnuABC_{At}$ systems are still functioning, it may need $ZinT_{At}$ and $YciC_{At}$ to effectively shuttle zinc ions to essential zinc-dependent proteins whose functions are required to cope with low-zinc stress.

Zinc uptake systems are known to be important for virulence in many bacteria (59, 60). However, in virulence assays using *N. benthamiana* as the host plant, growth either in LB (a zinc-replete condition) or in LB plus 0.5 mM EDTA (a zinc-depleted condition) had no effect on the virulence of *A. tumefaciens* mutant strains lacking zinc uptake genes compared to the wild type. Zinc availability at the infection site may differ between different types of plants, and this may alter the sensitivity of the assay in different host plants.

A model of zinc homeostasis in *A. tumefaciens* is proposed in Fig. 6C. Intracellular zinc levels are sensed by two transcriptional regulators, Zur and ZntR (25, 61). Zur is a repressor in the Fur family, whereas ZntR is an activator belonging to the MerR family. Under low-zinc conditions, Zur is in the apo form, which in turn triggers the expression of zinc acquisition genes, including *znuABC*, *troCBA*, *zinT*, and *yciC*. The TroCBA transporter plays a dominant role in zinc uptake compared to the ZnuABC system. ZinT may help enhance the efficiency of zinc recruitment to the TroCBA transporter. When TroCBA is impaired, the ZnuABC system plays an essential role in zinc uptake to support bacterial growth. It is likely that zinc can also be transported into the cytoplasm by an unidentified low-affinity zinc importer(s). The ZinT and YciC proteins may act as metallochaperones to allocate zinc to their target zinc-dependent proteins in the periplasm and cytoplasm, respectively. When intracellular zinc is overloaded, the complexes of Zn^{2+} with the zinc sensors, Zur-Zn^{2+} and ZntR-Zn^{2+}, are formed. The Zur-Zn^{2+} complex represses the zinc uptake genes, whereas the ZntR-Zn^{2+} complex activates the zinc efflux gene, *zntA*. Consequently, the high-affinity zinc uptake systems, TroCBA and ZnuABC, are shut down, and excess zinc can be pumped out of the cytoplasm by a P_{1B}-type ATPase, ZntA. However, the outer-membrane channels for zinc import and zinc extrusion in *A. tumefaciens* remain to be identified.

A. tumefaciens was shown here to operate a novel pathway for zinc uptake in which $ZinT_{At}$ is part of a high-affinity zinc importer, $TroCBA_{At}$. Whether $ZinT_{At}$ interacts with $TroA_{At}$ or directly interacts with the inner-membrane permease, $TroB_{At}$, during the process of zinc transport awaits further study. High-affinity zinc uptake systems, such as $TroCBA_{At}$ and $ZnuABC_{At}$, are necessary for *A. tumefaciens* to survive under low-zinc stress, but zinc allocation to zinc-dependent proteins facilitated by the zinc chaperones $ZinT_{At}$ and $YciC_{At}$ is also essential. Nevertheless, the exact molecular mechanisms of $ZinT_{At}$ and $YciC_{At}$, as well as their target proteins, have yet to be elucidated.

ACKNOWLEDGMENTS

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