Control of zinc homeostasis in *Agrobacterium tumefaciens* via *zur* and the zinc uptake genes *znuABC* and *zinT*

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The *Agrobacterium tumefaciens* zinc uptake regulator (*Zur*) was shown to negatively regulate the zinc uptake genes *znuABC*, encoding a zinc transport system belonging to the ATP-binding cassette (ABC) transporter family, and *zinT*, which encodes a periplasmic zinc-binding protein. The expression of *znuABC* and *zinT* was inducible when cells were grown in medium containing a metal chelator (EDTA), and this induction was shown to be specific for zinc depletion. The expression of *znuABC* was reduced in response to increased zinc in a dose-dependent manner, and *zinT* had a less pronounced but similar pattern of zinc-regulated expression. The inactivation of *zur* led to constitutively high expression of *znuABC* and *zinT*. In addition, a *zur* mutant had an increased total zinc content compared to the WT NTL4 strain, whereas the inactivation of *zinT* caused a reduction in the total zinc content. The *zinT* gene is shown to play a dominant role and to be more important than *znuA* and *znuB* for *A. tumefaciens* survival under zinc deprivation. ZinT can function even when ZnuABC is inactivated. However, mutations in *zur*, *znuA*, *znuB* or *zinT* did not affect the virulence of *A. tumefaciens*.

**INTRODUCTION**

Zinc is an essential trace element that is required for the growth and survival of bacteria. However, as zinc can be toxic to cells when a critical concentration is exceeded (Kasahara & Anraku, 1974; Singh & Bragg, 1974; Beard *et al.*, 1995; Aagaard & Brzezinski, 2001), the levels of intracellular zinc must be tightly regulated. Bacteria have mechanisms to maintain zinc homeostasis by controlling the delicate balance between zinc uptake (Hantke, 2005) and efflux systems (Blencowe & Morby, 2003; Nies, 2003, 2007), and they also have storage systems that safely collect zinc for use during zinc starvation (Nanamiya *et al.*, 2004; Akanuma *et al.*, 2006; Shin *et al.*, 2007; Gabriel & Helmann, 2009).

In bacteria, there are two major types of zinc import systems for high- and low-affinity zinc uptake (Hantke, 2005). In *Escherichia coli*, high-affinity zinc uptake is mediated via the co-operation of ZnuABC (zinc uptake) (Patzer & Hantke, 1998, 2000) and ZinT (formerly known as YodA) (Kershaw *et al.*, 2007; Graham *et al.*, 2009; Gabbianelli *et al.*, 2011). ZnuABC is an ABC transporter consisting of three proteins: the periplasmic protein ZnuA, which captures zinc and delivers it to the membrane permease ZnuB, and the ATPase ZnuC. ZinT protein was originally identified as a cadmium-induced periplasmic protein (Ferianc *et al.*, 1998), and ZinT was later shown to be a zinc-binding protein (Kershaw *et al.*, 2007; Graham *et al.*, 2009). The transcription of *znuABC* and *zinT* is upregulated under conditions of low zinc and in response to metal chelators, such as EDTA and N,N,N′,N′-tetrakis
Zinc uptake regulator (Zur) is a transcriptional regulator in the Fur (ferric uptake regulator) family and functions as a repressor of the furABC and Zur. Under high-zinc conditions, Zur complexes with Zn$^{2+}$ and binds to a conserved AT-rich sequence known as the Zur box (Panina et al., 2003), which is found in the promoter region of zinc uptake genes, thereby inhibiting gene expression to prevent an excessive amount of zinc in cells (Patzer & Hantke, 2000; Outten & O’Halloran, 2001). ZnuA and Zur are periplasmic proteins. The interaction of ZnT with the ZnuABC transporter has been demonstrated in Salmonella enterica serovar Typhimurium (Petrarca et al., 2010). Although ZnT is not absolutely required for the process of zinc uptake that is mediated by the ZnuABC system in Salmonella, the co-operation between ZnT and ZnuA may help to increase the uptake of zinc under severe zinc shortage and could benefit cell survival under variable environmental conditions (Petrarca et al., 2010). A study in E. coli O157:H7 showed that the functions of ZnT and ZnuA are not redundant (Gabbianelli et al., 2011); in addition, the apo-form of ZnT could be secreted outside the cells to capture environmental zinc (Gabbianelli et al., 2011).

Agrobacterium tumefaciens is a plant pathogen belonging to the alpha-proteobacteria and about which little is known regarding zinc homeostasis. In the present study, A. tumefaciens strains containing mutations in zur, znuA, znuB or zinT were generated to determine the gene functions involved in controlling the cellular zinc content. The zinc-responsive regulation of znuA, znuCB and zinT mediated by the transcriptional regulator Zur was investigated, and the differential roles of ZnuABC and ZinT were revealed.

**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 used were as previously described (Bhubhanil et al., 2014a). A. tumefaciens and E. coli were grown aerobically at 28 °C and 37 °C, respectively, in Luria–Bertani (LB) medium. In some experiments, AB medium (Cangelosi et al., 1991) was used as a minimal medium (containing per litre: K$_2$HPO$_4$, 3 g; NaH$_2$PO$_4$, 1.15 g; NH$_4$Cl, 1 g; MgSO$_4$7H$_2$O, 0.3 g; KCl, 0.15 g; CaCl$_2$, 0.01 g; FeSO$_4$7H$_2$O, 2.5 mg; glucose, 0.45%). Induction broth pH 5.5 (LB 5.5) (Cangelosi et al., 1991) was used in the virulence assay.

**Molecular techniques.** General molecular techniques were performed using standard protocols (Sambrook et al., 1989). Primers are listed in Table S1 (available in the online Supplementary Material). The sequence of cloned DNA was confirmed by DNA sequencing (Macrogen). Plasmid DNA was electroporated into A. tumefaciens strains (Cangelosi et al., 1991). All mutant strains were confirmed by Southern blot analysis.

**Construction of the A. tumefaciens zur mutant strain.** The zur gene (Atu1825) was disrupted by a single homologous recombination method (Ngok-Ngam et al., 2009). The internal coding region of the zur gene (Atu1518) was amplified with primers BT983 and BT984 and cloned into the unique Smal site of pKNOCK-Gm (Alexeyev, 1999), generating the plasmid pKNOCK2ZUR. The plasmid pKNOCKZUR was electroporated into WT NTL4, and the zur mutant (SPP12) was selected on LB agar (LA) plates containing 60 μg ml$^{-1}$ of gentamicin (Gm).

**Reverse transcription PCR (RT-PCR).** Total RNA was extracted from exponential-phase cells of WT NTL4 grown in LB medium, and RT-PCR was performed using previously described protocols (Ngok-Ngam et al., 2009). Primer sets for amplifying the junctions of znuC-znuB (BT4065 and BT4066) and znuB-zur (BT4048 and BT4049) were used in the PCRs with the following conditions: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 59.6 °C (znuC-znuB) or 59 °C (znuB-zur) for 20 s and extension at 72 °C for 30 s, with a final extension step at 72 °C for 5 min. DNA-contamination controls, in which reverse transcriptase (RT) was omitted, were run in parallel to ensure that there was no DNA contamination. The PCR products were visualized using gel electrophoresis through a 1.8% agarose gel with ethidium bromide staining. The experiment was repeated twice.

**Quantitative real-time PCR (qRT-PCR) analysis.** Exponential-phase cells grown in LB or minimal AB medium were either untreated or treated with various metals and a metal chelator (EDTA, 1 mM) for 15 min prior to harvest. The metals used were CdCl$_2$, CoCl$_2$, CuSO$_4$, FeCl$_3$, MnCl$_2$, NiCl$_2$ and ZnCl$_2$ at a final concentration of 1, 10, 50 or 750 μM. Total RNA was extracted and reverse transcribed to cDNA as described previously (Ngok-Ngam et al., 2009). qRT-PCR was performed according to a previous protocol (Bhubhanil et al., 2014b). DNA-contamination controls, in which RT was omitted, were also run in parallel. The gene-specific primers for znuA, znuB, znuC, zinT and 16S rRNA are listed in Table S1. The data are reported as the means of biological triplicates ± SD.

**Construction of znuA, znuB, zinT and double-mutant strains.** The A. tumefaciens znuA (Atu1521), znuB (Atu1519) and zinT (genes (BT1049) were individually disrupted. Primer pairs were used to amplify the internal coding regions of the znuA (BT3749 and BT3750), znuB (BT4134 and BT4135) and zinT genes (BT3747 and BT3748), and the PCR products were cloned into pKNOCK-Gm or pKNOCK-Km, generating plasmids pKNOCKZNUA, pKNOCKZNUB and pKNOCKZINT, respectively. The plasmids were electroporated into WT NTL4, and the znuA mutant (PS132), znuB mutant (PP1410) and zinT mutant (PC135) strains were selected on LA containing 60 μg ml$^{-1}$ of Gm or 30 μg ml$^{-1}$ of kanamycin (Km).

The AT1315 strain (disruption of both the znuA and zinT genes) was constructed by cloning the PCR fragment containing the znuA gene (BT3749 and BT3750) into pKNOCK-Km, generating the plasmid pKNOCKZNUA. The plasmid was electroporated into the mutant PC135 strain, and the mutant AT1315 was selected on LA containing 60 μg ml$^{-1}$ of Gm and 30 μg ml$^{-1}$ of Km.

The AB1410 (disruption of both the znuA and znuB genes) and BT1410 (disruption of both the znuB and zinT genes) strains were constructed by transferring the plasmid pKNOCKZNUB into the znuA mutant (PS132) and zinT mutant (PC135) strains, respectively.
Table 1. Strains and plasmids used in this study

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The double-mutation strains were selected on LA containing 60 μg ml⁻¹ of Gm and 30 μg ml⁻¹ of Km.

Inactivation of znuB did not inhibit transcription of the downstream gene zur according to an RT-PCR analysis (data not shown).

Cloning of functional zur, znuA and zinT genes for complementation. DNA fragments of full-length zur (BT982 and BT3804), znuA (BT3805 and BT3806) and zinT (BT3807 and BT3809) were amplified by PCR using Pfu DNA polymerase (Fermentas). The PCR products were cloned into SmaI-digested pBBR1MCS-4 (Kovach et al., 1995), generating pZUR, pZNUA and pZINT, respectively.

Measurement of total cellular metal content. Cells were grown in LB individually supplemented with 50 μM CdCl₂, CoCl₂, CuSO₄, FeCl₃, MnCl₂, NiCl₂ or ZnCl₂ or grown in AB medium in the absence or presence of 50 μM ZnCl₂. After incubation at 28 °C for 24 h, samples were prepared, and the metals were measured in parts per billion (p.p.b.) using an inductively coupled plasma mass spectrometer (ICP-MS), as described previously (Bhubhanil et al., 2014a). The data are reported as the means of biological triplicates ± SD.

Sensitivity to EDTA. A sensitivity test was performed using a dilution method (Ngok-Ngam et al., 2009). Cells grown in LB medium were adjusted, serially diluted and spotted onto AB, AB + 1.4 mM EDTA and AB + 1.4 mM EDTA + 0.1 mM ZnCl₂ plates. 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.

Virulence assay. A. tumefaciens strains carrying plasmid pCMA1 were used to infect young Nicotiana benthamiana plants according to a previously described protocol (Kamoun et al., 2003). Exponential-phase cells grown in LB were washed and resuspended in an IB 5.5 medium containing 300 μM acetosyringone (AS). The cells were incubated at 28 °C with shaking for 20 min, harvested and adjusted to an OD₆₀₀ of 1 in IB 5.5 + 300 μM AS. A 3 μl aliquot of the cell suspension was used to inoculate a wounded N. benthamiana petiole; 15 petioles were tested for each bacterial strain. Deformation of the petiole (crooked petiole phenotype, tumour formation) was observed at 4 weeks after inoculation.

RESULTS

A. tumefaciens zur is the repressor of zinc uptake genes

The A. tumefaciens C58 genome (Wood et al., 2001) contains putative high-affinity zinc uptake genes, znuABC and zinT (Fig. 1a). The zur (Atu1521) gene is oriented in the opposite direction to znuC (Atu1520) and znuB (Atu1519), and the transcriptional regulator zur-like gene (Atu1518) is located immediately downstream of the znuB gene (Fig. 1a). Sequence analysis suggested that znuC, znuB and zur could be co-transcribed. RT-PCR analyses were performed to confirm the transcriptional organization of...
these genes using RNA isolated from WT NTL4 and primer sets that amplify the junctions between znuC and znuB and between znuB and zur. The results confirmed that znuC, znuB and zur are co-transcribed (Fig. 1b).

Computational analysis of the A. tumefaciens genome identified Zur binding sites in the region between znuCBzur and znuA and in the promoter region of zinT (Atu1049) (Panina et al., 2003) (Fig. 1a). These data suggest that znuA, znuBCzur and zinT are under the control of the transcriptional regulator Zur. To determine whether these zinc uptake genes are regulated by Zur, a zur mutant strain (SPP12) was constructed, and the expression levels of znuA, znuB, znuC and zinT were determined using qRT-PCR. The zur mutant strain (SPP12/pBBR) exhibited higher expression of znuA (75-fold), znuB (3-fold), znuC (3-fold) and zinT (230-fold) than the WT (NTL4/pBBR) (Fig. 1c), and this de-repression of znuA, znuB, znuC and zinT was fully reversed in the complemented strain (SPP12/pZUR).
pZUR) (Fig. 1c). These results demonstrated that Zur is the transcriptional repressor of znuABC and zinT and that zur is auto-regulated.

**Induction of znuA, znuC and zinT in response to zinc-depleted conditions**

It was hypothesized that the expression of zinc uptake genes would be increased when cells are grown under zinc-limiting conditions. Thus, a qRT-PCR analysis was performed using RNA isolated from WT NTL4 grown in LB medium that was either untreated or treated with 1 mM EDTA (a metal-cation chelator). It was found that the expression of znuA, znuC and zinT increased by approximately 38-fold, 3-fold and 175-fold, respectively, in response to EDTA treatment when compared to untreated cells.

Next, the metal specificity of the transcriptional repression of znuA, znuC and zinT was determined. WT NTL4 was grown in LB medium containing 1 mM EDTA and supplemented with 0.75 mM of either CdCl2, CoCl2, CuSO4, FeCl3, MnCl2, NiCl2 or ZnCl2. The levels of EDTA-induced expression of znuA (Fig. 2a), znuC (Fig. 2b) and zinT (Fig. 2c) are presented as 100% of those under untreated conditions (LB, 2.6%, 29.5% and 0.57%, respectively). The results indicated that when compared to other metal ions (Cd2+, Co2+, Cu2+, Fe3+, Mn2+ and Ni2+), Zn2+ had the highest capacity to repress the EDTA-induced expression of znuA (Fig. 2a), znuC (Fig. 2b) and zinT (Fig. 2c). These results further support the view that znuABC and zinT are zinc uptake genes that respond to zinc deprivation.

**Differential repression of znuA, znuC and zinT in response to zinc-replete conditions**

To determine the effect of high concentrations of ZnCl2 on the expression of znuA, znuC and zinT, qRT-PCR was performed using RNA samples isolated from WT NTL4 grown in minimal AB medium supplemented with various concentrations of ZnCl2. The expression of znuA was reduced by approximately 40%, 70% and 75% with the addition of 1, 10 and 50 μM ZnCl2, respectively (Fig. 3a), and the expression of znuC was similarly reduced by 57%, 60% and 72% in response to an increasing Zn2+ concentration (Fig. 3b). In contrast, zinT expression was reduced by approximately 10%, 25% and 30% (Fig. 3c) in response to the addition of 1, 10 and 50 μM ZnCl2, respectively. These results demonstrated that znuA, znuC and zinT are negatively regulated by zinc; furthermore, znu is more sensitive than zinT to zinc levels.

**The zur mutant has increased total cellular zinc**

An ICP-MS analysis was performed to test whether the zur mutation affected the total cellular metal content. Cells were grown in LB medium individually supplemented with 50 μM CdCl2, CoCl2, CuSO4, FeCl3, MnCl2, NiCl2 or ZnCl2. The results showed that the zinc content in the zur mutant cells (SPP12/pBBR) was approximately fourfold higher than that in WT cells (NTL4/pBBR) (Fig. 4). Furthermore, this increased zinc content phenotype was reversed in the complemented strain (SPP12/pZUR) (Fig. 4). The zur mutant cells also exhibited, to a lesser extent, increased accumulation of Cd, Co, Cu, Fe and Ni (approx. <2.5-fold) (Fig. 4). In contrast, the Mn content in WT and the zur mutant was not significantly different. These results...
support the view that Zur is a repressor of the metal uptake genes znuABC and zinT, which are likely specific for zinc. Therefore, the loss of this repressor of zinc uptake could lead to a greater accumulation of zinc compared to other metals.

When considering uncontrolled zinc uptake genes (Fig. 1c) and the increased total zinc content (Fig. 4), one might expect the A. tumefaciens zur mutant strain to be more sensitive to high-zinc concentrations than the WT, as is observed in other bacteria (Tang et al., 2005; Yang et al., 2007; Feng et al., 2008; Dowd et al., 2012). Nonetheless, the growth of the A. tumefaciens zur mutant in LB containing high concentrations of zinc (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM ZnCl₂) was comparable to that of the WT cells (data not shown).

**The zinT mutant is more sensitive to zinc starvation mediated by EDTA**

In several bacteria, zinc uptake-deficient mutant strains exhibit a growth defect in zinc-depleted media (Lu et al., 1997; Lewis et al., 1999; Berducci et al., 2004; Yang et al., 2006; Ammendola et al., 2007; Davis et al., 2009; Desrosiers et al., 2010; Nielubowicz et al., 2010; Petrarca et al., 2010; Gabbianelli et al., 2011; Dowd et al., 2012). Therefore, we determined the growth of the WT and mutant strains on solid AB medium supplemented with 1.4 mM EDTA. The WT NTL4, SPP12 (zur mutation) and PS132 (znuA mutation) strains showed similar growth on AB+EDTA (Fig. 5a), suggesting that mutation of zur or znuA has no effect on the survival of A. tumefaciens under metal limitation. In contrast, PC135 (zinT mutation) and the double mutant AT1315 (znuA and zinT mutations) were approximately $10^3$-fold more sensitive to EDTA than the WT NTL4 strain (Fig. 5a), and this EDTA-sensitive phenotype of the zinT mutant was reversed in the complemented strain (PC135/pZINT, Fig. 5b). Furthermore, the addition of 0.1 mM ZnCl₂ also reversed the EDTA-sensitive phenotype of the zinT mutant (AB+EDTA+Zn, Fig. 5b), whereas the addition of other metals (CdCl₂, CoCl₂, CuSO₄, FeCl₃, MnCl₂ or NiCl₂) could not complement the phenotype (Fig. S1). These data demonstrated an important role for zinT in A. tumefaciens for surviving under zinc-limiting conditions.

As shown in Fig. 5a, the AT1315 strain (znuA and zinT mutations) did not show an increase in EDTA sensitivity when compared to the PC135 strain (zinT mutation), suggesting that the phenotypes of AT1315 was likely due to the loss of zinT and not znuA. This notion was supported by evidence that the EDTA-sensitive phenotype of AT1315 could be completely reversed by complementation with a plasmid carrying a functional zinT gene (pZINT) but not a functional znuA (pZNUA) gene (Fig. S2). Unlike zinT, the znuA gene did not play an apparent role under the tested conditions.

**Increased zinT expression in the znuA mutant strain**

The A. tumefaciens znuA mutant strain (PS132) showed no phenotype, raising the question of whether ZinT could compensate for the loss of ZnuA because both ZnuA and ZinT are predicted to be periplasmic zinc-binding proteins. To test this idea, the expression of zinT was determined in the znuA mutant and compared to that in WT using qRT-PCR. It was found that the znuA mutant had increased zinT expression that was approximately 3.5-fold higher than that of the WT (Fig. 5c). These results supported the idea that zinT could be upregulated to compensate for the loss of znuA.

Next, we determined the effect of zinT inactivation on znuA expression. It was found that the znuA expression levels in the zinT mutant (PC135) were not significantly

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**Fig. 3.** Differential repression of znuA, znuC and zinT by zinc. qRT-PCR analysis of znuA (a), znuC (b) and zinT (c). WT NTL4 cells grown in minimal AB medium were either untreated or treated with 1, 10 and 50 μM ZnCl₂ for 15 min. The levels of gene expression of the treated samples are presented as a percentage relative to that in the untreated sample (AB, 100%). The experiment was performed in biological triplicate, and bars and error bars indicate the mean and SD.
Fig. 4. The zur mutant shows increased accumulation of zinc. Cells were grown in LB medium individually supplemented with 50 μM CdCl₂, CoCl₂, CuSO₄, FeCl₃, MnCl₂, NiCl₂ or ZnCl₂ at 28 °C for 24 h. The total metal contents of the WT (NTL4/pBBR), zur mutant (SPP12/pBBR) and complemented (SPP12/pZUR) strains were measured using ICP-MS. The results are displayed as the means of biological triplicate samples, and error bars indicate SD. For comparison between NTL4/pBBR and SPP12/pBBR, the bars marked with * are significantly different (P<0.05 in an unpaired Student’s t-test).

Fig. 5. (a, b) The effect of EDTA on the growth of WT NTL4, the zur (SPP12), znuA (PS132) and zinT (PC135) single mutants and the znuA and zinT double mutant (AT1315). pBBR is the plasmid vector. Cells were complemented with a functional zinT or znuA gene on plasmid pZINT or pZNUA, respectively. Cells grown in LB medium were adjusted, serially diluted and spotted onto AB, AB + 1.4 mM EDTA or AB + 1.4 mM EDTA + 0.1 mM ZnCl₂ plates. Tenfold serial dilutions are indicated. (c) qRT-PCR analysis of zinT and znuA expression by NTL4, PC132 and PC135 cells grown in LB medium. The fold changes in gene expression in the mutant strains are relative to that of the WT NTL4. The experiment was performed in biological triplicate, and bars and error bars indicate the mean and SD.
different from those in WT (Fig. 5d). Therefore, zinT inactivation has no apparent effect on znuA expression.

**znuA and znuB are less important than zinT for A. tumefaciens for controlling zinc levels**

The lack of a phenotype for the A. tumefaciens znuA mutant strain (Fig. 5a) and the upregulation of zinT in the znuA mutant (Fig. 5c) raised the possibility of co-operation between A. tumefaciens ZinT and ZnuBC in zinc transport. To test this idea, a strain containing mutations in znuB and zinT was generated, and its sensitivity to EDTA was determined. The PC135 (zinT mutation) and BT1410 (znuB and zinT mutations) strains showed similar levels of EDTA sensitivity (Fig. 6a). The EDTA-hypersensitive phenotype of BT1410 could be fully reversed by complementation with plasmid pZINT (Fig. S3), suggesting that ZinT could function independently of ZnuABC. Furthermore, the single znuB mutation (PP1410) or double znuA and znuB mutations (AB1410) had no effect on EDTA sensitivity compared to that of WT (Fig. 6a). These results demonstrated that the znuA and znuB genes are less

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**Fig. 6.** (a) EDTA sensitivity test of WT NTL4, the znuA (PS132), znuB (PP1410), zinT (PC135) single mutants, the znuA and znuB double mutant (AB1410) and the znuB and zinT double mutant (BT1410). Cells grown in LB medium were adjusted, serially diluted and spotted onto AB and AB + 1.4 mM EDTA plates. Tenfold serial dilutions are indicated. (b) Total zinc contents of the WT NTL4, PS132 (znuA mutation), PP1410 (znuB mutation), PC135 (zinT mutation), AB1410 (znuA and znuB mutations), AT1315 (znuA and zinT mutations), BT1410 (znuB and zinT mutations) and SPP12 (zur mutation) strains were measured using ICP-MS. Cells were grown in minimal AB medium supplemented with 50 μM ZnCl₂ for 24 h. The results are displayed as the means of biological triplicate samples, and error bars indicate SD. For comparison with WT NTL4, the bars marked with * are significantly different (P<0.05 in an unpaired Student's t-test). (c) Virulence assay. A. tumefaciens cells carrying plasmid pCMA1 were grown in the IB 5.5 medium containing 300 μM acetosyringone (AS). Wounded petioles of N. benthamiana were inoculated with WT NTL4, SPP12 (zur mutation), AB1410 (znuA and znuB mutations), PC135 (zinT mutation) and BT1410 (znuB and zinT mutations). Fifteen petioles were tested for each bacterial strain, and tumour formation was observed at 4 weeks after inoculation. Representative petioles are shown. Control I: without inoculation. Control II: inoculation of wounded petiole with IB 5.5+300 μM AS.
important than 
for 
A. tumefaciens survival under zinc starvation.

The total zinc content was also measured using cells grown in minimal AB medium supplemented with 50 μM ZnCl_2. Mutations in znuA or znuB individually or in combination (PS132, PP1410 and AB1410, respectively) had no effect on the cellular zinc content, whereas mutation in zinT (PC135) led to a slight reduction in zinc content (Fig. 6b). PC135 (zinT mutation), AT1315 (znuA and zinT mutations) and BT1410 (znuB and zinT mutations) showed similar levels of zinc (P > 0.05 in an unpaired Student’s t-test), suggesting that znuA and znuB did not play an important role in controlling the cellular zinc content under the tested condition.

Ability of mutant strains to cause disease in plants

A. tumefaciens induces crown gall tumours on dicotyledonous plants by transferring T-DNA from the bacterium’s tumour-inducing (Ti) plasmid into the host plant cells (Zhu et al., 2000). Thus, a virulence assay was performed by inoculating N. benthamiana petioles with A. tumefaciens strains carrying a Ti plasmid (pCMA1). The plants infected with the mutant strains, SPP12 (zur mutation), AB1410 (znuA and znuB mutations), PC135 (zinT mutation) and BT1410 (znuB and zinT mutations) displayed tumour formation that was similar to the plants infected with WT NTL4 (Fig. 6c). These results suggested that zur, znuA, znuB and zinT are not essential for A. tumefaciens virulence during interaction with the host plant N. benthamiana.

DISCUSSION

It is demonstrated here that A. tumefaciens Zur negatively controls the zinc uptake genes znuABC and zinT (Fig. 1c). The zur gene is co-transcribed with znuCB, whereas znuA is transcribed opposite to znuCBzur (Fig. 1a, b). A putative Zur binding site is present within the 104 bp region between znuA and znuCBzur and is most likely responsible for the observed transcriptional regulation of these genes by Zur. In the A. tumefaciens zur mutant, de-repression of znuA transcription was much higher than that of znuCBzur (Fig. 1c), implying that the znuA promoter is more highly regulated than the znuCBzur promoter. The inactivation of A. tumefaciens zur led to the uncontrolled expression of zinc uptake genes and increased the accumulation of cellular zinc (Fig. 1c, Fig. 4). However, zur inactivation did not affect the ability of A. tumefaciens to survive in the tested high-zinc environment. One possible explanation was that the zinc accumulated in the A. tumefaciens zur mutant might be stored safely and is not toxic to the cell. The accumulation of cadmium, cobalt, copper, iron and nickel was also increased in the A. tumefaciens zur mutant (Fig. 4), suggesting that these metals may possibly be transported by the zur-regulated metal uptake systems.

Another possible explanation is that these metals are bound by proteins that would normally bind zinc.

The expression of A. tumefaciens znuA, znuC and zinT was upregulated specifically upon zinc depletion (Fig. 2) and was repressed in response to increased zinc levels (Fig. 3). Similarly to many other bacteria, the regulation of these zinc uptake genes is mediated by Zur, which belongs to the Fur family. Although Zur and Fur have similar structures, they respond specifically to Zn^{2+} and Fe^{2+}, respectively, and this metal selectivity results from differences in the coordination geometry of their regulatory metal-binding sites (Outten et al., 2001; Lucarelli et al., 2007). Zur has evolved to be a transcriptional regulator that specifically senses Zn^{2+} in the cell (Outten & O’Halloran, 2001) and represses zinc uptake genes under high-zinc conditions to prevent zinc-induced toxicity (Hantke, 2005). The repression of znuA and znuC transcription by zinc is greater than that of zinT (Fig. 3), suggesting that A. tumefaciens ZinT may have a housekeeping function in zinc uptake; in contrast, the ZnuABC system appears to assist in adjusting and maintaining proper intracellular zinc levels so as to not exceed cellular demand.

Salmonella ZnuA plays a dominant role in zinc transport, whereas ZinT is less important. The Salmonella ZnuA protein was detected at the high-zinc concentrations that completely inhibit Salmonella ZinT production (Petrarca et al., 2010). Furthermore, the growth impairment under EDTA treatment of the Salmonella znuA mutant was more severe than that which was observed for the Salmonella zinT mutant strain. znuA is required for the virulence of Salmonella (Campoy et al., 2002), but inactivation of zinT has no effect (Petrarca et al., 2010). Sequence alignment analyses showed that ZinT has high homology to the C-terminal domain of the AdcA protein, a component of an ABC-type zinc permease in Streptococcus pneumoniae (Dintilhac et al., 1997) and that the N-terminal domain of AdcA is similar to ZnuA. As shown in Salmonella (Petrarca et al., 2010), a complex between ZnuA and ZinT that resembles the structure of the AdcA protein can be formed in vitro. The ZnuA protein contains a His-rich loop which is inserted into the ZinT cavity when forming the ZnuA–ZinT complex in the presence of Zn^{2+} (Ilari et al., 2014). Loss of the His-rich loop led ZnuA to lose the ability to form a stable complex with ZinT in vitro (Ilari et al., 2014). However, whether the ZnuA and ZinT complex forms in vivo and functions similarly to AdcA is still not known.

In contrast to E. coli and Salmonella, the A. tumefaciens Znu system did not exhibit an apparent function under the tested conditions, whereas A. tumefaciens ZinT played a dominant role in survival under zinc shortage (Figs 5a, b, 6a). The inactivation of A. tumefaciens znuA led to the induction of zinT expression (Fig. 5c), and this zinT induction could have resulted from de-repression mediated by Zur when this repressor was in the apo-form in low intracellular zinc environments. Although our ICP-MS
analysis showed that the znuA mutant (PS132) did not have a total zinc content that was less than that of WT NTL4 (Fig. 6b), we could not rule out the possibility that the free zinc ions that were available to Zur in the znuA mutant may have been lower than that in the WT. It is also possible that zinT induction in the znuA mutant is mediated by an additional not-yet-identified mechanism.

The direct interaction between ZnuA and ZinT and the participation of ZinT in zinc uptake mediated by ZnuABC have been previously demonstrated in Salmonella (Petrarca et al., 2010). At present, there is no evidence supporting the hypothesis that A. tumefaciens ZnuA can interact with ZinT.

It appears that the roles of ZnuABC and ZinT and their contributions to the control of zinc homeostasis are different among bacteria. Unlike many other bacteria, A. tumefaciens ZinT was shown to play a more important role than ZnuA and ZnuB in controlling zinc homeostasis. Moreover, A. tumefaciens ZinT was able to function independently of ZnuABC (Fig. S3). ZinT could be a versatile protein that functions in zinc uptake, and could be a zinc-binding chaperone that either provides zinc to other zinc transport systems or delivers the metal to zinc-containing enzymes in the periplasm (Hantke, 2005). The loss of A. tumefaciens ZinT but not ZnuA and ZnuB, caused a slight reduction in the cellular zinc content (Fig. 6b), implying the existence of an additional high-affinity zinc uptake system or a compensatory effect due to other multiple metal ion transporter(s) such as ZurAM, ZinABC, ZevAB and TroABCD. Listeria monocytogenes contains two zinc uptake systems, ZurAM and ZinABC, which are regulated by Zur (Corbett et al., 2012). ZurAM and ZinABC are related ABC-type zinc permease systems and their functions are redundant. The presence of these two zinc uptake systems helps L. monocytogenes respond to diverse zinc environments during different stages of infection (Corbett et al., 2012). In addition to ZnuABC, Haemophilus influenzae has a second zinc uptake system named ZevAB. H. influenzae requires ZnuABC for optimal growth at a wide range of zinc levels, while ZevAB is particularly essential for growth under severe zinc shortage (Rosadini et al., 2011). The TroABCD system in Treponema pallidum and Streptococcus suis is capable of transporting Mn2+ and Zn2+ (Desrosiers et al., 2007; Zheng et al., 2011). At present, the functions of A. tumefaciens genes homologous to either zurAM, zevAB or annotated genes belonging to the Tro-like family (such as Atu4498-Atu4499 and Atu3178-Atu3179-Atu3180) have not been investigated. Recently, the siderophore, yersiniabactin (Ybt), has been reported to be a zincophore for Zn2+ acquisition in Yersinia pestis (Bobrov et al., 2014). Ybt is secreted to capture Zn2+ from the environment. The Ybt-Zn2+ complex is imported into the cytoplasm via the inner-membrane protein, YbtX, which belongs to the major facilitator superfamily. The Ybt system could compensate for the loss of ZnuABC; however, both systems contribute to successful infection by Y. pestis (Bobrov et al., 2014). The existence of a Ybt-like system in A. tumefaciens remains to be determined. Controlling zinc uptake and zinc efflux both help to maintain the intracellular zinc homeostasis (Blencowe & Morby, 2003; Nies, 2003, 2007; Hantke, 2005), and the A. tumefaciens C58 genome (Wood et al., 2001) contains zntA (Atu0843) and zntB (Atu0731) genes, which encode putative zinc exporters that have yet to be characterized. Therefore, further identification of additional zinc uptake systems and investigation of these zinc efflux systems are needed to gain a better understanding of how A. tumefaciens maintains zinc homeostasis.

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