Roles of *Agrobacterium tumefaciens* C58 ZntA and ZntB and the transcriptional regulator ZntR in controlling Cd\(^{2+}\)/Zn\(^{2+}\)/Co\(^{2+}\) resistance and the peroxide stress response

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The putative zinc exporters ZntA (a P\(_{1B}\)-type ATPase) and ZntB (2-TM-GxN family) in *Agrobacterium tumefaciens* were characterized. The expression of the *zntA* gene is inducible by CdCl\(_2\), ZnCl\(_2\) and CoCl\(_2\), of which CdCl\(_2\) is the most potent inducer, whereas *zntB* is constitutively expressed. The metal-induced expression of *zntA* is controlled by the MerR-like regulator ZntR. The *zntA* and *zntR* mutants were highly sensitive to CdCl\(_2\) and ZnCl\(_2\), and CoCl\(_2\) sensitivity was demonstrated to a lesser extent. By contrast, the *zntB* mutant showed similar levels of metal resistance to the WT strain. Even in the *zntA* mutant background, *zntB* did not play an apparent role in metal resistance under the conditions tested. The inactivation of *zntA* increased the accumulation of intracellular cadmium and zinc, and conferred hyper-resistance to H\(_2\)O\(_2\). Thus, the metal transporter ZntA and its regulator ZntR are important for controlling zinc homeostasis and cadmium and cobalt detoxification. The loss of either the *zntA* or *zntR* gene did not affect the virulence of *A. tumefaciens* in *Nicotiana benthamiana*.

INTRODUCTION

Zinc plays an essential role in structural protein stabilization and as a cofactor for the catalytic activity and regulatory function of many enzymes and proteins. However, at high concentrations, zinc competes with other metal ions for active sites, generating non-functional proteins and thereby inhibiting many vital biological processes. Zinc homeostasis in bacteria is maintained through the regulation of the uptake, efflux and storage of zinc (Blencowe & Morby, 2003; Nies, 2003, 2007; Nanamiya et al., 2004; Hantke, 2005; Akanuma et al., 2006; Shin et al., 2007; Gabriel & Helmann, 2009). Zinc uptake genes are inducible under low-zinc conditions. When zinc levels surpass cellular demand, the zinc uptake regulator (Zur) represses the zinc uptake genes, *znuABC* and *zinT*, to prevent excess zinc-mediated toxicity (Patzer & Hantke, 1998; Petrarca et al., 2010). Furthermore, expression of the zinc efflux gene *zntA* is activated via the Zn\(^{2+}\)-responsive transcriptional regulator (ZntR), which maintains intracellular zinc at suitable levels in *Escherichia coli* (Brocklehurst et al., 1999; Singh et al., 1999). ZntA is likely not the only Zn\(^{2+}\) efflux pump in *Escherichia coli*, which also possesses *zntB*. In *Salmonella enterica* serovar Typhimurium, *zntB* is known to encode a protein belonging to the 2-TM-GxN family (Knoop et al., 2005), which can mediate efflux of Zn\(^{2+}\) and Cd\(^{2+}\) ions (Worlock & Smith, 2002).

An association between zinc homeostasis and oxidative resistance has been reported (Gaballa & Helmann 2002; Yang et al., 2007; Smith et al., 2009; Cerasi et al., 2014; Sein-Echaluce et al., 2015). Zinc serves as a cofactor for oxidant-detoxifying enzymes. Zinc protects thiol groups from free radicals and inhibits free radical formation by...
competing with redox-active metals, such as copper and iron. A role for zinc in the protection against metal-mediated oxidative damage has been demonstrated (Korbashi et al., 1989; Har-el & Chevion, 1991). The deregulation of zinc transport affects the bacterial response to peroxide stress. The overexpression of Zur from cyanobacterium Anabaena sp. PCC 7120 confers H$_2$O$_2$ resistance (Gaballa & Helmann, 2002). The expression of zurA (zinc uptake under oxidative stress) gene (Gaballa & Helmann, 2002). The expression of zurA is controlled by the peroxide-sensing transcriptional regulator PerR. The induction of zurA under H$_2$O$_2$ stress elevates zinc levels, which in turn might protect thiol from oxidation (Gaballa & Helmann, 2002). The zurA homologue pmtA (PerR-regulated metal transporter) was identified in Streptococcus pyogenes (Brenot et al., 2007). In contrast to zurA, pmtA might function as a zinc exporter and play a role in resistance to zinc toxicity (Brenot et al., 2007). The pmtA mutant strain exhibits H$_2$O$_2$ hypersensitivity (Brenot et al., 2007). These studies demonstrate that, in bacteria, zinc uptake and zinc export genes could play roles in H$_2$O$_2$ resistance via different mechanisms.

A. tumefaciens, a member of alphaproteobacteria, is a soil bacterium causing crown gall tumour disease in dicotyledonous plants (Zhu et al., 2000). The regulation of the A. tumefaciens C58 zinc uptake genes znuABC and zntT via Zur has been previously reported (Bhubhanil et al., 2014c). Mutation of the A. tumefaciens zur gene increased the expression of znuABC and zntT and increased the accumulation of intracellular zinc (Bhubhanil et al., 2014c). Herein, we aimed to assess the physiological roles of the genes annotated zntR (Atu0888), zntA (Atu0843) and zntB (Atu0731), which may be involved in controlling zinc efflux in A. tumefaciens C58 (Wood et al., 2001). ZntA is important for A. tumefaciens survival under high-zinc conditions, whereas ZntB plays no role in zinc resistance under the conditions tested. The control of zinc levels and H$_2$O$_2$ stress response through Zur via zinc uptake and ZntR via zinc efflux was also investigated.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in the present study are shown in Table 1. A. tumefaciens and E. coli were aerobically grown at 28 and 37 °C, respectively, in Luria–Bertani (LB) medium. LA refers to LB medium containing 1.5 % agar. The growth conditions and antibiotic concentrations used in the present study have been previously described (Bhubhanil et al., 2014a). Minimal AB medium (containing 1%): 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 %] and induction broth, pH 5.5 (IB 5.5), were prepared as previously reported (Bhubhanil et al., 2014a; Cangelosi et al., 1991).

**Molecular techniques.** General molecular techniques were performed using standard protocols (Sambrook et al., 1989). The primers are listed in Table S1, available in the online Supplementary Material. DNA sequencing was performed to confirm the sequence of the cloned DNA (Macrogen). Plasmid DNA was transferred into A. tumefaciens strains by electroporation (Angelosi et al., 1991).

**Construction of zntR, zntA, zntB and double-mutant strains.** The mutant strains were generated using a single homologous recombination method (Ngok-Ngam et al., 2009). The A. tumefaciens zntR (Atu0888), zntA (Atu0843) and zntB (Atu0731) genes were individually disrupted. Primer pairs for gene inactivation (Table S1) were used to amplify the internal coding regions of zntR, zntA and zntB, and the PCR products were cloned into pKNOCK-Km, generating pKNOCKmZNTR, pKNOCKmZNTA and pKNOCKmZNTB, respectively. The plasmids were electroporated into the WT strain, and the zntR (ZR1423), zntA (ZA141) and zntB (ZB141) mutant strains were selected on LA containing 30 μg ml$^{-1}$ kanamycin (Km).

To generate the ZAB141 strain (disruption of both zntA and zntB genes), the PCR fragment corresponding to the internal coding region of zntB was cloned into pKNOCK-Gm, generating the plasmid pKNOCKZNTB. The plasmid was electroporated into the mutant ZA141 strain, and the mutant ZAB141 was selected on LA containing 60 μg ml$^{-1}$ gentamicin (Gm) and 30 μg ml$^{-1}$ Km.

To generate the ZRA15 strain (disruption of both zntR and zntA genes), the PCR fragment corresponding to the internal coding region of zntA was cloned into pKNOCK-Gm, generating the plasmid pKNOCKZNTA. The plasmid was electroporated into the mutant ZR1423 strain, and the mutant ZRA15 strain was selected on LA containing 60 μg ml$^{-1}$ Gm and 30 μg ml$^{-1}$ Km. All the mutant strains were confirmed through Southern blot analysis.

**Construction of plasmids expressing functional zntR, zntA and zntB genes.** DNA fragments of full-length zntR, zntA and zntB were PCR amplified using genomic WT DNA as a template, gene-specific primer pairs for complementation (Table S1) and Pfu DNA polymerase (Fermentas). The PCR products were cloned into Smal-digested pBR1MCS-4 (Kovach et al., 1995), generating the plasmids pZNTR, pZNTA and pZNTB, respectively.

**Quantitative real-time PCR (qRT-PCR) analysis.** qRT-PCR was performed as previously described (Bhubhanil et al., 2014b). Exponential-phase cells grown in LB were either untreated or treated with various metals for 15 min prior to harvest. The metal salts CdCl$_2$, CoCl$_2$, CuCl$_2$, FeCl$_3$, MgCl$_2$, MnCl$_2$, NiCl$_2$ and ZnCl$_2$ were used at a final concentration of 100, 250, 500 or 750 μM. The gene-specific primers for zntA, zntB and 16S rRNA are listed in Table S1. The relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method. The fold-changes in gene expression were relative to the untreated control as previously described (Livak & Schmittgen, 2001). The data are reported as the means of biological triplicates ± s.d.

**Determination of the transcriptional start site for zntA using 5’ rapid amplification cDNA ends (5’ RACE).** The transcriptional start site of zntA was determined using RNA samples isolated from WT exponential-phase cells grown in LB and treated with 750 μM ZnCl$_2$ for 15 min, followed by 5’RACE (Roche), according to the manufacturer’s instructions. Specific primers SP1 and SP2 correspond to BT4122 and BT4822, respectively.
Table 1. Strains and plasmids used in this study

<table>
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Ap<sup>+</sup>, ampicillin resistance; Gm<sup>+</sup>, gentamicin resistance; Km<sup>+</sup>, kanamycin resistance.

**Sensitivity to metals.** The dilution method (Ngok-Ngam et al., 2009) was used for the metal sensitivity test. Exponential-phase cells grown in LB medium were adjusted, serially diluted and spotted onto plates containing LA, LA + CdCl<sub>2</sub> (25, 50, 350, 400 and 450 μM), LA + CoCl<sub>2</sub> (0.75, 1 and 1.25 mM) and LA + ZnCl<sub>2</sub> (350, 400 and 450 μM). The plates were subsequently incubated at 28 °C for 48 h. Each strain was examined in duplicate, and each experiment was repeated at least twice.

**Measurement of the total cellular metal content.** Cells were grown in LB individually supplemented with 10 μM CdCl<sub>2</sub> and 50 μM CoCl<sub>2</sub>, CuSO<sub>4</sub>, FeCl<sub>3</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub> or ZnCl<sub>2</sub> at 28 °C for 24 h. The cells were washed three times with 50 mM potassium phosphate buffer, pH 7.0 (KPB), and 10 mM EDTA. The cells were subsequently washed twice with 50 mM KPB and resuspended in KPB to achieve OD<sub>600</sub> 1. The cell suspension (2.5 ml) was used for sample preparation, and the metals were measured using an inductively coupled plasma mass spectrometer (ICP-MS), as previously described (Bhubhanil et al., 2014a). The data were reported as the means of biological triplicates ± SD.

**Sensitivity to H<sub>2</sub>O<sub>2</sub> and diamide.** Exponential-phase cells grown in LB medium were adjusted, serial-diluted and spotted onto plates containing LA or LA + 275 μM H<sub>2</sub>O<sub>2</sub> in the absence or presence of 50 μM ZnCl<sub>2</sub> and LA + diamide (600, 700 and 800 μM). The plates were incubated at 28 °C for 48 h. Each strain was examined in duplicate, and each experiment was repeated at least twice.

**Catalase activity assay.** Exponential-phase cells grown in LB medium were either left untreated or treated with 500 μM H<sub>2</sub>O<sub>2</sub> for 30 min. Crude bacterial lysates were subsequently prepared, and catalase activity was determined based on the degradation of H<sub>2</sub>O<sub>2</sub> at a wavelength of 240 nm using a previously described protocol (Beers & Sizer, 1952; Kitphati et al., 2007). One unit (U) of catalase was defined as the amount of enzyme capable of catalysing the turnover of 1 μmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> under the assay conditions. Specific activity was calculated in U (mg protein)<sup>-1</sup>. The data are reported as the means of biological triplicates ± SD.

**Virulence assay.** A. tumefaciens strains carrying the plasmid pCM1 were used to infect young Nicotiana benthamiana plants according to a previously described protocol (Kamoun et al., 2003; Bhubhanil et al., 2014c). Exponential-phase cells grown in LB were washed and grown in an IB 5.5 medium containing 300 μM acetosyringone (AS) for another 20 min. A 5 μl aliquot of the cell suspension (OD<sub>600</sub> 1 in IB 5.5 + 300 μM AS) was used to inoculate a wounded N. benthamiana petiole. Fifteen petioles were tested for each bacterial strain. Tumour formation was observed at 4 weeks after inoculation.

**RESULTS**

**zntR mediates the induction of zntA in response to cadmium, zinc and cobalt.** The expression of the putative zinc exporter genes zntA and zntB in response to zinc levels was determined using
quantitative real-time PCR (qRT-PCR) analysis. In the WT strain, the expression of zntA, but not zntB, was increased after treatments with 100, 500 and 750 μM ZnCl₂ (Fig. 1a). The zinc-mediated induction of zntA was abolished in the zntR mutant strain (ZR1423), suggesting that zntR activates zntA (Fig. 1a). The loss of zinc-induced zntA expression was able to be restored in the complemented strain (ZR1423/pZNTR) (Fig. S1a). Next, the metal-specific response of zntA was determined using WT cells grown in LB individually supplemented with 250 μM CdCl₂, CoCl₂, CuSO₄, FeCl₃, MgCl₂, MnCl₂, NiCl₂, PbSO₄ or ZnCl₂. The results shown in Fig. 1b suggest that Cd was the best inducer (~100-fold), followed by Zn (~14-fold) and Co (~8-fold), while other metals induced less than 3-fold zntA expression (Ni and Pb) or had no effect (Cu, Fe, Mg and Mn). The induction of zntA through Cd and Co was also abolished in the ZR1423 strain (data not shown). The expression of zntB was not affected by the zntR mutation (Fig. S1a), and zntB expression was not strikingly changed upon treatment with zinc (Fig. 1a) or other metals (Fig. S1b).

Fig. 1. (a) Transcription analyses of zntA and zntB in response to increased zinc levels using qRT-PCR. WT and zntR mutant (ZR1423) cells were grown to exponential phase in LB medium supplemented with 100, 500 and 750 μM ZnCl₂ for 15 min. The expression of the target genes was normalized to 16S rRNA, and the fold-changes in gene expression were assessed relative to WT cells grown in LB (regarded as 1). The experiment was performed in biological triplicate, and the error bars indicate SD. (b) qRT-PCR analysis of zntA expression in response to various metals. WT cells were grown in LB medium to exponential phase and individually supplemented with 250 μM CdCl₂, CoCl₂, CuSO₄, FeCl₃, MgCl₂, MnCl₂, NiCl₂, Pb(NO₃)₂ or ZnCl₂ for 15 min. The fold-changes in gene expression were assessed relative to cells grown in LB (regarded as 1). (c) Features of the A. tumefaciens zntA promoter. The ATG start codons for zntA and Atu0844 are indicated in bold. The transcriptional start site of zntA, determined through 5′ RACE, is indicated with an asterisk, and the corresponding guanine (G) is indicated in bold. The predicted −35 and −10 sequences are shown in bold. The dyad sequence has 6 bp of perfect symmetry (arrows) separated by 3 bp, indicated with a grey box. The putative ribosome-binding site (RBS) is also indicated.
The A. tumefaciens zntA promoter (PzntA<sub>A</sub>)

To identify the promoter region of zntA<sub>A</sub>, we used 5’RACE to determine the transcriptional start site of zntA<sub>A</sub>, and the −35 and −10 sequences were predicted using BPROM (www.softberry.com). The features of PzntA<sub>A</sub> are shown in Fig. 1c. The 5’RACE results indicated that the zntA<sub>A</sub> transcript starts at the G residue located 24 bp upstream of the ATG start codon. The predicted −10 sequence (CAGATT) and −35 sequence (TTGAAG) share 3/6 and 4/6 bp identity, respectively, with the consensus E. coli −10 sequence (TATAAT) and −35 sequence (TTGACA). The spacing between the predicted −10 and −35 boxes was 19 bp, compared with the 16–18 bp spacing of the consensus E. coli σ<sup>70</sup> promoter sequences (Harley & Reynolds, 1987). The dyad-symmetrical DNA sequence (the 6-3-6 bp inverted repeat, CTCTAG-TTG-CTAGAG) identified within the 19 bp spacer of PzntA<sub>A</sub> could be a potential binding site for the zntA regulator ZntR.

ZntR and ZntA are required for resistance to cadmium, zinc and cobalt

Next, the zntR (ZR1423) and zntA (ZA141) mutant strains were generated, and the metal sensitivity was determined (Fig. 2). The results showed that the ZR1423 strain was ~100-fold more sensitive to 50 μM CdCl<sub>2</sub> and 450 μM ZnCl<sub>2</sub> than WT (Fig. 2). The loss of zntA severely affected cell tolerance to cadmium and zinc. The ZA141 strain was ~10<sup>5</sup>-fold more sensitive to 450 μM ZnCl<sub>2</sub> than WT, while a low concentration (50 μM) of CdCl<sub>2</sub> completely inhibited the growth of ZA141 (Fig. 2). Furthermore, the strains ZR1423 and ZA141 also exhibited ~10-fold more sensitivity to CoCl<sub>2</sub> than WT (Fig. 2). The sensitive phenotypes of the zntR (ZR1423/pBBR) and zntA (ZA141/pBBR) mutant strains to CdCl<sub>2</sub>, ZnCl<sub>2</sub> and CoCl<sub>2</sub> could be reversed in the complemented strains (ZR1423/pZNTR and ZA141/pZNTA) (Fig. S2). However, the resistance to other metal salts (CuSO<sub>4</sub>, FeCl<sub>3</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub> and PbSO<sub>4</sub>) was similar in WT and the mutants ZR1423 and ZA141 (data not shown). These results demonstrated that A. tumefaciens zntR and zntA are important for the detoxification of cadmium, zinc and cobalt. In addition, strain ZRA15 (zntR and zntA mutations) did not show increased metal sensitivity when compared with ZA141 (zntA mutation) (Fig. S2), suggesting that the phenotype of ZRA15 was likely due to the loss of zntA. This notion was supported by the fact that the metal-sensitive phenotype of ZRA15 could be completely reversed by complementation with zntA carried on the plasmid pZNTA (Fig. S2).

ZntB plays no role in zinc resistance under the conditions tested

It has been reported that S. enterica serovar Typhimurium ZntB mediates the efflux of Zn<sup>2+</sup> and Cd<sup>2+</sup>, and the zntB mutant shows increased sensitivity to zinc and cadmium (Warlock & Smith, 2002). The single inactivation of A. tumefaciens zntB (ZB141) had no effect on cell tolerance to cadmium, zinc, cobalt (Fig. 2) and other metal salts (CuSO<sub>4</sub>, FeCl<sub>3</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub> and PbSO<sub>4</sub>) (data not shown). The metal sensitivity was also tested in the double-mutation strain (ZAB141) and the WT strain expressing multiple copies of zntB (WT/pZNTB). Mutations at zntB and zntA (ZAB141) showed no additional effect on the sensitivity to cadmium, zinc and cobalt compared with the single zntA mutation (ZA141) (Fig. 2). Moreover, the WT/pZNTB strain showed resistance to cadmium, zinc and cobalt similar to the WT strain carrying the empty vector (data not shown). Under the conditions tested, A. tumefaciens zntB did not show an apparent role for metal resistance.

The zntA mutant showed a striking increase in the accumulation of cadmium and zinc

ICP-MS analysis was performed to determine the metal content in the WT and mutant strains (Fig. 3). The cells were grown in LB medium individually supplemented with 50 μM CoCl<sub>2</sub>, CuSO<sub>4</sub>, FeCl<sub>3</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub> or ZnCl<sub>2</sub>. However, CdCl<sub>2</sub> was used at 10 μM owing to the Cd-hypersensitivity of the mutant strains (ZR1423, ZA141 and ZAB141; Fig. 2). The zur (zinc uptake regulator) mutant strain (SPP12) (Bhurbanil et al., 2014c) was used as a control, which showed the increased accumulation of zinc (~5-fold; Fig. 3). The inactivation of zntR (ZR1423) led to the increased accumulation of Cd (~1.5-fold), while the levels of other metals were similar to those in WT (Fig. 3). The loss of zntA increased the accumulation of Cd (~4-fold), Zn (~2-fold) and Mn (~1.2-fold) compared with WT, and the WT showed higher levels of Co (~1.6-fold) and Fe (~1.2-fold) than the zntA mutant (ZA141) (Fig. 3). The zntB mutation had a lesser effect on the metal content than the zntA mutation, as only the Cu and Mn contents in the zntB mutant (ZB141) were slightly changed relative to WT (Fig. 3). In addition, the accumulation of Ni in all of the mutants and WT was similar. The striking increase in the accumulation of Cd and Zn in the zntA mutant suggests that ZntA is an exporter of Cd and Zn ions.

Roles of zinc uptake and export systems in H<sub>2</sub>O<sub>2</sub> resistance

Zinc-mediated uptake of H<sub>2</sub>O<sub>2</sub> stress has been reported in B. subtilis (Gaballa & Helmann, 2002) and S. enterica (Cerasi et al., 2014). Thus, to examine whether the disruption of the zinc exporter or deregulation of zinc uptake in A. tumefaciens affected the ability of cells to survive under H<sub>2</sub>O<sub>2</sub> stress, the H<sub>2</sub>O<sub>2</sub> sensitivity test was performed. The ZR1423, ZA141 and SPP12 strains displayed more tolerance to H<sub>2</sub>O<sub>2</sub> than WT (LA + H<sub>2</sub>O<sub>2</sub> and LA + H<sub>2</sub>O<sub>2</sub> + ZnCl<sub>2</sub>; Fig. 4a). Furthermore, the survival of A. tumefaciens cells under H<sub>2</sub>O<sub>2</sub> stress was increased in the presence of ZnCl<sub>2</sub>. As hyper-resistance to H<sub>2</sub>O<sub>2</sub> in these mutants could also reflect increased catalase levels, the catalase activity assay was performed. However, as shown in Fig. S3a, the catalase levels in the mutant strains were not elevated compared...
with WT. It was possible that increased intracellular zinc levels might influence the H$_2$O$_2$ resistance observed in the mutant strains. To determine whether zinc plays a protective role against H$_2$O$_2$ stress in A. tumefaciens through the protection of protein thiols from oxidation, sensitivity to the thiol-oxidizing agent diamide was examined. The results showed that WT and the mutant strains showed similar levels of diamide resistance (Fig. S3b). Therefore, the mechanism for enhanced H$_2$O$_2$ resistance in the A. tumefaciens mutants (ZR1423, ZA141 and SPP12) remains unknown.
The inactivation of either zntR or zntA did not affect the virulence of *A. tumefaciens*

*A. tumefaciens* causes crown gall disease through the insertion of T-DNA from the tumour-inducing (Ti) plasmid into the plant genome (Zhu et al., 2000). The virulence of the mutant strains (ZR1423 and ZA141) compared with WT was examined after infecting *N. benthamiana* petioles. The results showed that tumour formation on *N. benthamiana* petioles infected with the mutant strains and WT was similar (Fig. 4b). These results suggested that zntR and zntA are not important for *A. tumefaciens* virulence in infecting the *N. benthamiana* host plant.

**DISCUSSION**

It has been reported that ZntA (a P1B-type ATPase) and ZntB (a transporter belonging to the 2-TM-GxN family) are zinc exporters (Knoop et al., 2005; Smith et al., 2014). In addition to Zn\(^{2+}\), *E. coli* ZntA (ZntAEc) extrudes Cd\(^{2+}\), Co\(^{2+}\) and Pb\(^{2+}\) (Beard et al., 1997; Rensing et al., 1997, 1998; Binet & Poole, 2000), *Staphylococcus aureus* ZntA (ZntASa) exports Co\(^{2+}\) (Xiong & Jayaswal, 1998), and *S. enterica* ZntB (ZntBSa) mediates the efflux of Cd\(^{2+}\) (Worlock & Smith, 2002). The genomic context of *A. tumefaciens* zntR, zntA and zntB is shown in Fig. S4. The expression of zntA*

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**Fig. 3.** Determination of metal contents using ICP-MS. WT, ZR1423 (zntR mutation), ZA141 (zntA mutation), ZB141 (zntB mutation) ZAB141, (zntA and zntB mutations) and SPP12 (zur mutation) cells were grown in LB medium individually supplemented with 10 \(\mu\)M CdCl\(_2\) and 50 \(\mu\)M CoCl\(_2\), CuSO\(_4\), FeCl\(_3\), MnCl\(_2\), NiCl\(_2\) or ZnCl\(_2\) at 28 °C for 24 h. The total metal contents were measured using ICP-MS. The results are shown as the means of biological triplicate samples, and the error bars indicate SD. The bars marked with an asterisk are significantly different from WT (\(P<0.05\) in an unpaired Student’s *t*-test). p.p.b., Parts per billion.
was inducible with the following order of effectiveness: CdCl₂ > ZnCl₂ > CoCl₂ (Fig. 1b). The *A. tumefaciens* zntA mutant was slightly sensitive to CoCl₂ but highly sensitive to CdCl₂ and ZnCl₂ (Fig. 2), suggesting that ZntAAt might be involved in the export of these metals. The ICP-MS analysis showed that the *A. tumefaciens* zntA mutant apparently accumulated Cd²⁺ and Zn²⁺, but not Co²⁺, to levels higher than WT (Fig. 3), further suggesting that ZntAAt plays a role in exporting Cd²⁺ and Zn²⁺, rather than Co²⁺. In contrast to ZntAAt, ZntBAt did not show an apparent role for metal resistance under the conditions tested using various strains, including the single zntBAt mutation (Fig. 2) and the expression of multiple copies of zntBAt from a plasmid in the WT background (data not shown). Furthermore, inactivation of zntBAt in the zntAAt mutant background (ZAB141) showed levels of metal resistance similar to the zntAAt mutant (ZA141) (Fig. 2).

The CorA protein, a member of the 2-TM-GxN family, facilitates Mg²⁺ uptake (Smith & Maguire, 1998; Knoop et al., 2005) and mediates Co²⁺ and Ni²⁺ uptake (Snayley et al., 1989). Although *S. enterica* serovar Typhimurium ZntB (ZntBSt) is a distant CorA homologue (Knoop et al., 2005) (Fig. S5), this protein does not transport Mg²⁺ (Worlock & Smith, 2002). Sensitivity to magnesium, cobalt and nickel was examined to determine whether zntBAt is involved in the transport of these metals. However, WT and the zntBAt mutant strain showed similar resistance to magnesium, cobalt and nickel (data not shown). *A. tumefaciens* contains a gene annotated corA (Atu0710) (Fig. S5); however the gene function has not been investigated. The signature motifs of CorA (YGMNFxxMPEL) and ZntB (GxxG[D/V]NxGGxP) contribute differently to metal selectivity (Knoop et al., 2005). ZntBAt contains the GxxGMNxDExP motif, which differs from the highly conserved G[I/V]N and GG dipeptide, resulting in the classification of ZntBAt in a different subclass from ZntBSt, which contains the GxxGVNxGGxP motif (Knoop et al., 2005) (Fig. S5). The zntBAt gene is flanked upstream by mmgC (Atu0732), encoding an acyl-CoA-dehydrogenase (Fig. S4). Downstream of zntBAt is a gene (Atu0730) encoding a lectin-like protein (Fig. S4). ZntBAt shares 17 % amino acid identity with ZntBSt (Fig. S5). The C94 and C307 residues in the ZntBSt protein may be involved in Zn²⁺ transport (Wan et al., 2011). In ZntBSt, the residues corresponding to C94 and C307 in ZntBSt are replaced with D94 and S311, respectively (Fig. S5). The signature motif sequence and corresponding metal-specific-binding residues in ZntBAt are different from those in ZntBSt, implying that ZntBAt might serve a different function, which remains elusive. The expression of zntBAt in response to metals was not dramatically changed (Fig. S1b) compared with zntAAt expression (Fig. 1b). It is possible that ZntBAt could be a metal transporter with broad specificity. However, ICP-MS analysis revealed that the zntBAt mutation showed only a minor effect on the accumulation of metals in the cells grown in LB

![Fig. 4. (a) Sensitivity to H₂O₂. WT, ZA141 (zntA mutation), ZR1423 (zntR mutation) and SPP12 (zur mutation) cells grown in LB medium were adjusted, serially diluted and spotted onto plates containing LA, LA + 275 μM H₂O₂ and LA + 275 μM H₂O₂ + 50 μM ZnCl₂. Tenfold serial dilutions are indicated, and the plates were incubated at 28 °C for 48 h. (b) Virulence assay using young *N. benthamiana* plants. *A. tumefaciens* strains carrying the plasmid pCMA1 were grown in IB 5.5 medium containing 300 μM AS and used to inoculate wounded *N. benthamiana* petioles. Fifteen petioles were tested for each bacterial strain. 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.](http://mic.sgmjournals.org)
supplemented with metals (Fig. 3). In addition, the lack of a phenotype in the *A. tumefaciens* zntB mutant might reflect the functional redundancy of zntB with zntA and other putative cation diffusion facilitator genes, such as Atu0891, Atu0991 and Atu2274, that have not yet been characterized (Cubillas et al., 2013). ZntBAt is a homologue of the metal importer CorA (Smith & Maguire, 1998; Knoop et al., 2005). We could not rule out the possibility that ZntBAt may function as a metal importer. To test this idea, ICP-MS analysis was performed using cells grown in LB and minimal AB medium (Fig. S6). However, the results showed that the Cu content in the zntB mutant (ZB141) changed only slightly relative to WT when cells were grown in LB (Fig. S6). It is possible that the function of ZntBAt may be masked by functional redundancy with other metal transporters, and thus was not revealed under the test conditions.

In *E. coli* and *S. aureus*, zntA is under the control of ZntR, but acts through different mechanisms to activate zntA expression in response to increased zinc levels (Brocklehurst et al., 1999; Singh et al., 1999). *E. coli* ZntR (ZntREc) is a transcriptional activator belonging to the MerR family, while *S. aureus* ZntR (ZntRSA) is a transcriptional repressor belonging to the ArsR/SmtB family. ZntRSA binds to the imperfect 9–2–9 bp inverted repeat (ATATGAGAA-AA-TATTCCATAT) in the zntRSA promoter region, and this interaction is inhibited in the presence of Zn2+ (Singh et al., 1999). Apo-ZntREc binds to the perfect 11–11 bp inverted repeat (ACTCTGAAGTC-GACTCCA-GAGT) in the zntREc promoter region (Brocklehurst et al., 1999). ZntREc acts as an activator upon binding to Zn2+, which mediates zntAEC transcription via a MerR-like DNA distortion mechanism (Outten et al., 1999). Similar to *E. coli*, metal-induced zntAAt expression is mediated by the activation of zntAAt (Fig. 1a). The zntAAt promoter contains a 19 bp spacer between the −35 and −10 sequences (Fig. 1c) compared with the *E. coli* σ70 consensus promoter sequences, which are spaced 16–18 bp apart (Harley & Reynolds, 1987). The 19 bp extended spacer is a characteristic of the metal-ion-responsive MerR family promoters, including the Tn501 mer (Lund et al., 1986), *E. coli* zntA (Brocklehurst et al., 1999), *E. coli* copA (Stoyanov et al., 2001) and *Cupriavidus metallidurans* pbrA (Hobman et al., 2012) promoters. It has been shown that the 19 bp spacer between −35 and −10 sequences is essential for both normally weak levels of activity and the induction of the mer (mercury resistance) promoter through MerR (Parkhill & Brown, 1990). MerR mediates both the repression and activation of mer through MerR binding and Hg-MerR untwisting of the spacer region (Ansari et al., 1995). The expression of the copper exporter copA and the lead exporter pbrA is regulated by the MerR family transcriptional activators CueR and PbrR, respectively. MerR (7–4–7 bp, TCCGTAC-ATGA-GTACCGA), CueR (7–7–7 bp, ACCTCC-CCTTGCT-GGAAGGT) and PbrR (7–1–7 bp, CTATAGT-A-CTAGAG) bind to different dyad symmetrical DNA sequences within the 19 bp spacer sequence in the respective regulated promoters (Lund et al., 1986; Stoyanov et al., 2001; Hobman et al., 2012).

In contrast to the *E. coli* zntA promoter (11–11 bp inverted repeat), the *A. tumefaciens* zntA promoter contains a 6–3–6 bp inverted repeat (CTCTAG-TTG-CTAGAG) within the 19 bp spacer (Fig. 1c). ZntRAa shares 34% amino acid identity with ZntREc (Fig. S7). It has been shown that cyanisteine (C79, C114, C115, C124 and C141) and histidine (H29, H53, H76, H77 and H119) residues are important for the ZntREc-mediated regulation of zntA expression (Khan et al., 2002). Mutations at these residues induce changes in metal-ion preference, sensitivity and induction magnitude. The H29, H53 and H119 residues corresponding, respectively, to E29, E53 and H116 in ZntRAa are likely involved in either zinc ligation or DNA-bend modulation (Khan et al., 2002). C79, C114 and C124, corresponding to C78, C113 and C121, respectively, in ZntRAa are conserved metal-binding residues belonging to the MerR family (Fig. S7). The presence of a characteristic 19 bp spacer in the zntAAt promoter (Fig. 1c) and the conserved residues in the ZntRAa protein (Fig. S7) suggest that ZntRAa might activate zntAAt transcription through a mechanism similar to that of ZntREc and the MerR family regulators (Ansari et al., 1995; Outten et al., 1999; Stoyanov et al., 2001; Hobman et al., 2012).

The loss of zntAAt (ZIR1423) increased the sensitivity to CdCl2, ZnCl2 and CoCl2 compared with WT (Fig. 2). Compared with the zntAAt mutant (ZA141), the zntAAt mutant (ZIR1423) showed similar levels of CoCl2 tolerance, but higher levels of resistance to CdCl2 and ZnCl2. This finding might reflect the fact that zntAAt activation is impaired in the ZIR1423 strain. As zntAAt might play a major role in the efflux of cadmium and zinc, the complete loss of zntAAt (ZA141) showed a more severe effect on metal resistance. This notion was supported by evidence that the zntAAt mutant accumulated higher levels of Ca2+ and Zn2+ than the zntAAt mutant (Fig. 3). Interestingly, the zntAAt mutant was more sensitive to CoCl2 (Fig. 2) but had reduced intracellular Co2+ (Fig. 3) relative to WT. Therefore, the cobalt hypersensitive phenotype of the zntAAt mutant was not due to the increased levels of intracellular Co2+. The cobalt hypersensitivity might be an indirect effect resulting from a disruption of metal homeostasis affecting metals that are not specific substrates for ZntAAt.

The zinc content in the zurAt mutant was approximately 5-fold higher than that in the WT (Fig. 3), consistent with the previous report. However, the zurAt mutant showed levels of zinc resistance similar to WT (0.5–4 mM ZnCl2) (Bluhbanil et al., 2014c). In contrast, compared with WT, the zurAt mutant possessed an approximately 2-fold higher zinc content (Fig. 3) and showed high sensitivity to zinc (0.35–0.45 mM ZnCl2) (Fig. 2). Unlike the zntAAt mutant, it is likely that zinc is kept safely in the zurAt mutant and does not damage the cell.

The disruption of either zinc uptake or zinc export affects intracellular zinc levels and might be involved in peroxide resistance (Gaballa & Helmann, 2002; Brenot et al., 2007;
membrane-bound ferritin (MbfA) in iron transport and resistance (ZA141) increased intracellular zinc (Fig. 3) and enhanced H₂O₂ resistance (Fig. 4a). Zinc might confer protection against H₂O₂ stress in *Agr. tumefaciens*, although the exact mechanism remains unknown. Oxidative burst is an initial plant defence mechanism (Wojtaszek, 1997; Fones & Preston, 2013). The inactivation of the zinc exporter provided a benefit that would help *A. tumefaciens* to cope with H₂O₂ stress (Fig. 4a); thus, it was not surprising that *A. tumefaciens* virulence was fully retained in the zinc-exporter-defective mutants (ZR1423 and ZA141, Fig. 4b).

In conclusion, the *A. tumefaciens* zntA gene is an important metal exporter for the detoxification of cadmium, zinc and cobalt. The regulation of zinc homeostasis by uptake systems via Zur and the exporter ZntA via ZntR plays a role in the ability of *A. tumefaciens* to survive H₂O₂ stress.

**ACKNOWLEDGEMENTS**

The authors thank S. K. Farrand for the pCMA1 plasmid and P. Srifah Huehne and K. Bhinija for technical assistance with the virulence assay. The authors also thank P. Sittipo and S. Bhubhanil for assistance with the experiments. This work was financially supported by a grant from the Chulabhorn Research Institute and the Thailand Research Fund (no. RSA5880010) to R. S.

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**SUPPLEMENTARY MATERIAL**


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Edited by: G. Thomas