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Zinc dependence of *zinT* (*yodA*) mutants and binding of zinc, cadmium and mercury by ZinT

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Abstract

ZinT (B1973), previously known as YodA, was originally characterised as a cadmium-induced periplasmic protein under the regulation of Fur and SoxS. Here we describe a decrease in *zinT* transcript in response to elevated copper concentrations and the zinc and copper dependent phenotype of a Δ*zinT* strain. Cadmium sensitivity of the Δ*zinT* strain was not observed. We demonstrate the binding of nickel, zinc, cadmium, and mercury, but not cobalt, copper, iron, and manganese, to purified ZinT using mass spectrometry. This and previous studies support the hypothesis that ZinT plays a role in zinc homeostasis and is required for growth under zinc limited conditions, suggesting that ZinT is either a periplasmic zinc chaperone or is involved in zinc import. Limited metal ion discrimination results in regulation of *PzinT* in a non-specific manner, which is mirrored in the binding of several different heavy metals by ZinT.

Keywords: ZinT; YodA; Zinc import; Cadmium; Mercury

Metals are essential for all living organisms. They provide the basis for the function of a wide range of proteins which are involved in reduction and oxidation reactions, and contribute to the structural integrity of others [1]. Metals can be categorised into two groups: essential and non-essential. Non-essential metals have no known beneficial biological function and at elevated concentrations several (e.g., mercury, cadmium and lead) compete with essential metals (e.g., zinc, calcium) for functional groups in proteins. Essential metals have important biological functions, but at high intracellular concentrations they can be toxic, as they can displace other essential metals or participate in deleterious chemical reactions. Some redox-active metal ions, such as copper and iron, can generate reactive oxygen species that damage cellular components. Therefore, the concentrations of essential metals in the cell need to be tightly regulated [2].

To achieve this, *Escherichia coli* contains a number of regulators and structural proteins that respond to external metal ion concentrations. One such protein is ZinT (previously known as YodA/B1973) [3]. ZinT is a 23 kDa protein that has been characterised as a member of a cadmium-stress stimulon. Under cadmium stress ZinT expression is induced and the protein is localised to the cytoplasm, but after prolonged exposure to cadmium, periplasmic ZinT can be observed [4]. This periplasmic localisation is thought to involve the cleavage of a 24 amino acid (aa) leader sequence to leave a polypeptide of 190 amino acids [4]. ZinT is structurally related to the lipocalin/calycin family of proteins, which are often implicated in the transport of small hydrophobic molecules [5].

Initially, the expression of *zinT* was shown to be strongly down-regulated in *E. coli* strains possessing a H-NS mutation [6]. More recently it has been shown that expression from P*zinT* is induced by both cadmium and

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hydrogen peroxide and that this induction is dependent on *soxS* and *fur* and influenced by *relA* and *spoT* [7]. Bioinformatic analysis of DNA sequences upstream of *zinT* has identified a putative Zur binding site in the upstream region of *zinT* suggesting possible zinc regulatory effects [3].

The three-dimensional structure of ZinT has been elucidated in the presence of bound cadmium and zinc cofactors [5]. Cadmium is coordinated by three histidine residues, his^{144}, his^{153}, and his^{155}. In addition to these three histidine residues, glu^{189} and his^{193} are involved in the binding of two zinc ions. When the native form of the protein was purified without any added metals, a metal ion (possibly zinc, copper or iron) occupied the central metal binding site that binds cadmium [5].

Amino acid alignment (Supplementary Fig. 1) also shows how this protein is conserved in other bacterial species, including Gram-positive bacteria. The histidine-rich metal-binding centre of ZinT is similar to the C-terminal domain of AdcA, a periplasmic binding protein of the zinc ABC transport system, in *Streptococcus pneumoniae* [8]. This protein is conserved throughout the *streptococci* and although it is much larger (423 amino acids) than ZinT, the C-termini appear highly conserved between the two proteins [9]. A mutation in the *adc* operon of *S. pneumoniae* results in a requirement for zinc to restore growth equivalent to that of the wild type strain [10].

In this paper, we describe the construction of a Δ*zinT* mutant and the effects of different metal ion concentrations and other stresses on this mutant. We also describe metal ion binding to purified ZinT analysed by mass spectroscopy. These data suggest that ZinT is involved in periplasmic zinc binding and either the subsequent import or shuttling of zinc to periplasmic zinc-containing proteins under zinc-limited conditions.

Materials and methods

Strains and growth conditions. Escherichia coli K-12 MG1655 Seq (CGSC 7740) [11] was used throughout this study. Growth experiments were performed in Luria–Bertani Medium (LB) [12] or Defined Medium A (DMA), (11.3 g K_2PO_4, 5.4 g NaH_2PO_4, 200 mg $MgSO_4$, 10 mg $CaCl_2$, 5 mg $FeSO_4$, 0.5 mg $ZnSO_4$, 0.5 mg $MnSO_4$, 0.1 mg $CuSO_4$, 0.1 mg $CoCl_2$, 0.1 mg $Na_2B_4O_7$, 0.1 mg Na_2MoO_4, 0.26 g EDTA, 2 g NH_4Cl per litre) [13]. To minimise the amount of metal added to DMA as contaminants of the media components, Aristar (VWR) grade potassium and sodium phosphates were used. DMA was supplemented with 0.4% (w/v) glucose. Bacterial cultures were grown in sterile plasticware.

Growth experiments were performed in triplicate. A 5 μl inoculum from a stationary phase culture, grown either in DMA without copper supplementation or in LB, was added to 5 ml of growth medium in a sterile plastic universal bottle. These cultures were grown for 10 h at 37 °C with shaking (150 rpm); 300 μl samples were withdrawn at two hourly intervals and the OD_{595} was measured in a 96-well microtitre plate using a Labsystems Multiskan MS plate reader. LB was used when creating electrocompetent cells and for culturing prior to protein purification.

Semi quantitative real-time reverse transcriptase PCR. Real-time PCR was performed using SYBR green master mix (Applied Biosystems). Primers were added to a final concentration of 50 nM and 1 ng of cDNA was added. The cDNA used was produced using Superscript II $RNaseH^-$ reverse transcriptase (Invitrogen) from the RNA samples isolated as described previously [14]. Primers were designed using Primer Express software (supplied with the ABI PRISM™ 7000 sequence detection system; Applied Biosystems) to have a $T_m = 60 \pm 1$ °C and generate an amplicon of 101 nucleotides. These primers were RT F 5′-TTTT CGCATGGTCATCACTCAC-3′ and RT R 5′-AGCGTTCGGTTT TGTACATTGG-3′.

Construction of the ΔzinT mutant strain. Deletion mutations were made in *E. coli* MG1655 as described by Datsenko and Wanner [15]. The *zinT* mutant was produced by replacing *zinT* with a copy of the chloramphenicol acetyl transferase (*cat*) gene amplified from the plasmid pKD3 using primers: ZinT F 5′-ATTCGTCTTTACAAACTGGCTGTTGCTT TAGGTGTCTTTATTGTTATGTAGGCTGGAGCTGCTTCG-3′ and ZinT R 5′-CAATGAGACATCATTTCCTCGACCACTTCTTCGCGA ATATCCTCCTTAG-3′.

Cloning and protein purification. The *zinT* gene was amplified by PCR, using primers: '*zinT* cloning F' 5′-GGGGATCCTGGAGGAAACT GTT-3′ and '*zinT* cloning R′ 5′-GGGAGCTCTCAATGAGACATCA-3′, and cloned into pUTS18 [16] in front of an isopropyl β-thiogalactopyranoside (IPTG)-inducible promoter, P_{tac}, using the BamHI and SacI restriction sites. The sequence of the recombinant plasmid was confirmed by colony PCR, using a primer specific to pUTS18 (5′-GAG CTGTTGACAATTAATCATCGG-3′) and primer '*zinT* cloning R′, and sequencing of the colony PCR products (data not shown). Protein was purified from 1 L of cells, induced with 0.5 mM IPTG when $OD_{600} = 0.3$ and further cultured overnight at 30 °C with shaking at 150 rpm. Cells were harvested by centrifugation at 4400*g* for 30 min, resuspended in 20 ml of binding buffer (50 mM ammonium acetate, pH 7.9, 1 mM β-mercaptoethanol and 10 mM imidazole) and lysed using a Thermo spectronic French® pressure cell where the pressure at the aperture was 3000 psi. Cell debris was removed by centrifugation at 25,400*g* for 30 min. The resulting supernatant was applied to a 10 ml column containing nickel chelate agarose resin (Qiagen). Once the protein was bound, the column was washed with 50 ml of binding buffer followed by a 100 ml gradient of 10–250 mM imidazole in the same buffer, to elute the protein. Fractions (5 ml) were collected and those containing the ZinT protein were identified by polyacrylamide gel electrophoresis. These were dialysed overnight against three changes of 1 L of dialysis buffer (50 mM ammonium acetate, pH 7.9, and 1 mM β-mercaptoethanol) at 4 °C. The protein was then concentrated using Centriplus YM-10 columns (Amicon) and stored at 4 °C. The concentration of the purified ZinT was estimated spectrophotometrically using Coomassie reagent [17].

Metal binding analysis. ZinT (380 μM) was presented with 0.1, 0.01, or 0.001 molar equivalents of cadmium, cobalt, copper, iron, manganese, mercury, nickel and zinc salts in dialysis buffer to a final volume of 20 μl and incubated at room temperature for 2 h. To determine relative affinities of ZinT for zinc, cadmium, and mercury 0.1 mM ZinT was incubated with 1, 2, 5, 10, and 20 molar equivalents of each metal. Electrospray mass spectroscopy (Micromass, Manchester, UK) was used to determine the metal binding by the purified protein. Samples were injected at a flow rate of 200 μl/min in an electrospray carrier solution containing acetonitrile (50%) and formic acid (0.5%). Electrospray conditions were: capillary voltage at 3200 V, sampling cone voltage at 34 V, nitrogen as the carrier gas, and 300 °C. Mass was determined by time of flight mass spectroscopy. The analyzer was operated in positive-ion mode calibrated with Cytochrome *c*. A scan speed of 1 s/scan over the *m/z* (mass to charge ratio) range of 600–2000 produced mass spectral data. Scans were averaged and the final spectrum was deconvoluted using Maximum Entropy Software (Mass Lynx version 3.4). The purified protein without added metal was used as a control.

Results

Expression of zinT is repressed by increasing external copper

In a previous study [7], the authors noted that there was no transcriptional response of P*zinT* to elevated levels of copper, zinc, cobalt, and nickel. Recently, genome-wide

transcriptional profiling [18] has shown that *zinT* expression in *E. coli* K-12 increases in response to addition of the cell-permeable divalent metal ion chelator TPEN, which primarily chelates zinc, suggesting that expression of ZinT may be dependent on limiting levels of metals. We had previously seen that *zinT* expression decreased under increasing levels of copper in genome-wide transcriptional profiles [14], but the *zinT* transcription data had not passed all of the statistical analyses so was excluded from the final gene lists. We have now quantified P*zinT* response to copper-limited conditions when cultured in defined media. Semi-quantitative real-time reverse transcriptase (RT) PCR was performed on the RNA samples analysed in a previous microarray experiment [14] to obtain an expression profile for *zinT* in response to growth in DMA without copper supplementation or supplemented with 0.75 or 2 mM copper, supplied as copper–glycine at pH 7. The real-time RT-PCR data are expressed in relation to a pooled RNA sample [14] isolated from five separate *E. coli* cultures grown in DMA without copper supplementation. The expression of *zinT* decreases in response to increased copper (Fig. 1), however the fold decrease is greater in response to 0.75 mM copper–glycine than to 2 mM copper–glycine. This is similar to the expression of *znuA* in response to these copper concentrations [14].

Growth of the ΔzinT mutant is not sensitive to cadmium but is copper- and zinc-dependent

The *zinT* disruption mutant was made as described in Materials and methods and was confirmed by colony PCR and Southern blotting (data not shown). Initial investigations had suggested that *zinT* expression may be regulated as part of a more general stress response mechanism [7]. To test this, growth of the *zinT* disruption mutant and the parental strain were compared under different stress conditions: LB broth with addition of one of 1 mM paraquat, 5 mM hydrogen peroxide or 4% NaCl, pH 5, at 37 °C, or grown at 45 °C. Under the conditions tested, there was no significant difference in growth between the parental strain and *zinT* disruption mutant (Supplementary Fig. 2), suggesting that ZinT was not primarily involved in these generalised stress responses. Puskarova et al. [7] described P*zinT* induction in response to cadmium and suggested that ZinT is part of a cadmium stress response. To test whether ZinT contributed to tolerance to cadmium, the growth of the *zinT* disruption mutant was compared to the growth of the parental strain in LB broth supplemented with 0.25, 0.5 and 1 mM cadmium (Fig. 2A). No significant difference between the parental strain and the *zinT* disruption mutant in response to increased concentrations of cadmium was observed.

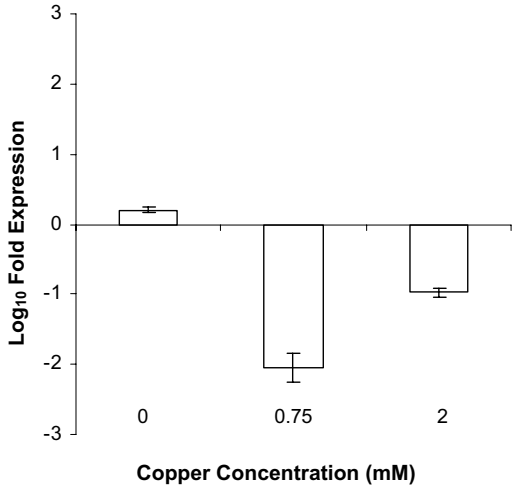

Fig. 1. Semi-quantitative real-time PCR analysis of the expression profile of *zinT* in response to no added copper, 0.75 mM copper–glycine and 2 mM copper–glycine. Data are shown as relative expression compared to a pooled RNA sample (no added copper). Error bars indicate the standard error of the mean.

We observed reduced levels of *zinT* transcript in response to elevated copper concentrations, which suggested a possible role in copper import. To test this, the *zinT* mutant strain was grown in DMA without copper supplementation or supplemented with 0.2, 0.4, 0.6, 0.8 or 1 mM copper–glycine (Fig. 2B). The *zinT* mutant strain required at least 0.6 mM copper–glycine for efficient growth but at higher concentrations (0.8 and 1 mM) the mutant grew less well than the parental strain.

ZinT bears a C-terminal similarity to AdcA of *S. pneumoniae*, a protein involved in zinc import [10], ZinT binds zinc [5] and there is a predicted Zur binding site upstream of the *zinT* promoter [3]. We investigated the effect of zinc concentrations upon growth of the *zinT* mutant and the parental strain, which were grown in LB supplemented with 0.5 and 1 mM zinc. There was no significant difference in growth between the parental strain and the *zinT* disruption mutant in response to increased concentrations of zinc in LB (Supplementary data). When the mutant and parental strain were grown under zinc-limited conditions the *zinT* mutant grew less well than the parental strain in DMA supplemented with zinc concentrations below 0.4 mM (Fig. 2C). When grown in DMA at elevated zinc concentrations (0.6, 0.8, and 1 mM) the *zinT* disruption strain grew better than the parental strain. Growth of the mutant strain was also retarded by elevated mercury concentrations compared to the parental strain (data not shown).

Purified ZinT binds nickel, zinc, cadmium and mercury

ZinT was purified using a naturally occurring N-terminal motif HGHHSH which is exposed after removal of the 24 amino acid leader sequence [19]. Mass spectrometry was used to identify metals bound by ZinT, by mass difference from the native protein. Previous MALDI-TOF mass spectrometric analysis of ZinT determined the molecular mass as 22.3 kDa [19]. In this investigation, the mass of ZinT was assessed using electrospray ionisation-mass spectrometry (ESI-MS).

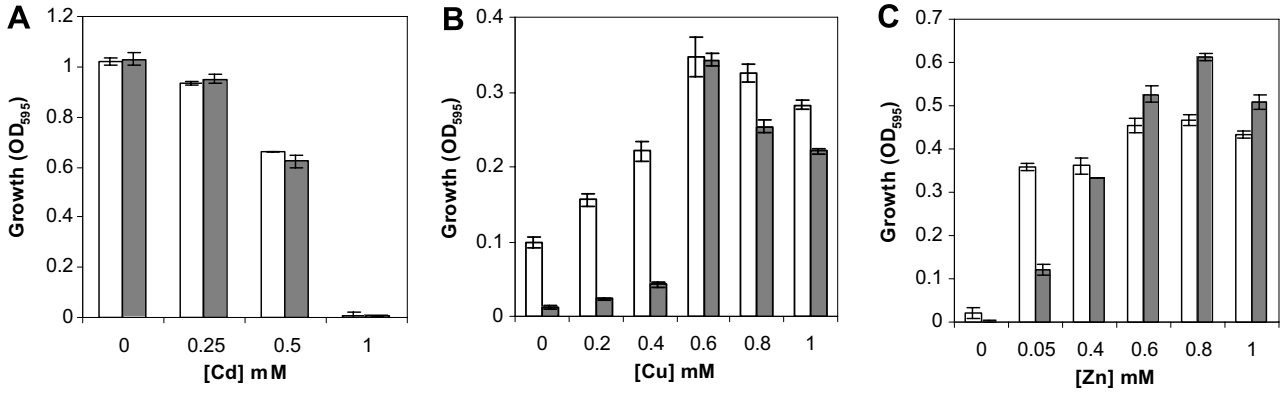

Fig. 2. Comparison of the growth of *E. coli* MG1655 and a MG1655 *zinT* disruption mutant in media supplemented with cadmium, copper or zinc. The growth of the *zinT* mutant strain (grey bars) was compared to the parental strain (white bars) in LB supplemented with cadmium (A) or DMA supplemented with copper (B) or zinc (C). Growth is shown as final OD_{595} after 7 h (Cd) or 10 h (Cu and Zn). Error bars indicate the standard error of the mean.

Cadmium, cobalt, copper, iron, manganese, mercury, nickel and zinc salts at 0.1, 0.01, or 0.001 molar equivalents were incubated with ZinT to determine qualitative aspects of metal binding. All metal ions had chloride as the counter ion. Only cadmium, mercury, nickel, and zinc binding were observed. It is possible that nickel binding was conferred by the N-terminal histidine residues used to purify ZinT [19]. Further experiments were performed to investigate the relative affinities of ZinT for the Group 12 transition metals; zinc, cadmium and mercury. To ZinT 1, 2, 5, 10, and 20 molar equivalents of cadmium, mercury or zinc were added and the protein–metal mixture was incubated for 2 h at room temperature in dialysis buffer. Binding of zinc and mercury was observed at 0.5, 1, and 2 mM (Fig. 3), but not at lower concentrations (data not shown). Cadmium binding was observed down to 0.1 mM, the lowest cadmium concentration investigated.

Discussion

Originally ZinT was characterised as a member of a cadmium stress stimulon [7], however, the growth of a *zinT* mutant is unaffected by cadmium, casting doubt over the possible role of ZinT in protecting cells from cadmium stress alluded to in the original studies [4,7]. The expression of *zinT* was also described as increasing in response to increased hydrogen peroxide concentrations in the growth media, leading the authors to postulate that ZinT may be involved in an oxidative stress response [7]. Our data show that the Δ*zinT* mutant was no more sensitive to hydrogen peroxide or paraquat than the parental strain, but our data do support a previously suggested, possible cellular role for ZinT, that of a zinc chaperone [20].

The transcriptional response of *zinT* to elevated concentrations of zinc, copper, cobalt and nickel had previously been investigated, but no transcriptional response to increased external concentrations of these metals was found [7]. By using a chemically defined medium, with controlled metal ion concentrations, it was possible to elucidate further potential functions of ZinT. The reduction in expression of *zinT* in response to copper supplementation, coupled with the growth phenotype of the mutant strain suggested a possible role in copper uptake, although this is not supported by *in vitro* metal binding assays, which failed to show copper binding by ZinT. The dependence of the Δ*zinT* mutant strain on zinc for growth and the binding of zinc by purified ZinT suggest that it may function in zinc homeostasis. The improved growth of the *zinT* mutant compared to the parental strain at higher concentrations of zinc, but not copper, support the hypothesis that this protein is required for growth in zinc-limited conditions. Cells in which *zinT* is deleted can therefore tolerate higher external zinc concentrations than the parental strain as less zinc is taken up. ZinT is not unique in this role, as other proteins have been implicated in zinc import. The Znu ABC transporter [21] and ZupT [22] have both been shown to import zinc, with ZupT having a lower affinity for zinc than ZnuABC. It has been proposed that the high affinity ZnuABC import mechanism may reduce freely available periplasmic zinc at low zinc concentrations, and that a zinc chaperone is required; this may be a function of ZinT [20]. Therefore, ZinT may be a zinc chaperone rather than another zinc import protein.

The mode of *zinT* regulation is unclear. Originally it was shown that H-NS was required for *zinT* expression and that cadmium induction of *zinT* is dependent on *soxS* and *fur*. However, a predicted Zur binding site has been observed upstream of *zinT* [3] and regulation of P*zinT* by Zur has been confirmed [18,20]. ZinT production under zinc-limited conditions [20] has also been reported. The apparent regulation of transcription of P*zinT* in response to copper is similar to the previously reported expression profile of *znuA* [14], a member of a zinc import pathway, suggesting that this regulation may be an indirect response to increased copper concentration, caused by Zur binding copper at high copper concentrations.

This study, along with others [4,7,18,20], shows that *zinT* transcription responds to, and its gene product binds

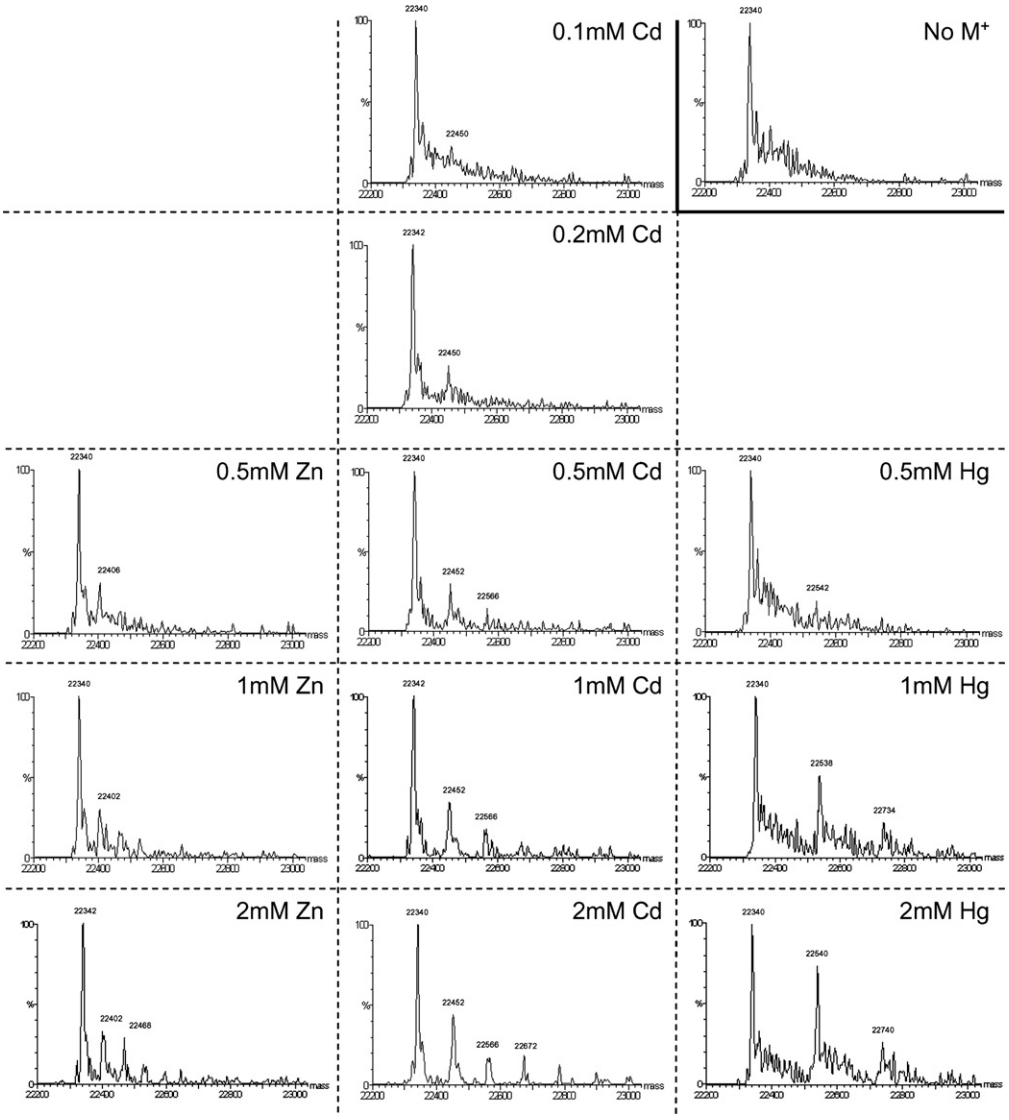

Fig. 3. Binding of zinc, cadmium and mercury by ZinT. ZinT (0.1 mM) was presented with 0.1, 0.2, 0.5, 1, or 2 mM of zinc or cadmium or mercury. Spectra are labelled with the metal ion and concentration used. ZinT without metal is indicated (No M^+). Binding of metal to ZinT (22340 Da) is indicated by an increase in mass (*x*-axis) by 65 Da (zinc), 112 Da (cadmium), or 201 Da (mercury) or multiples thereof. To simplify these data, spectra are omitted where no binding was observed.

to, different metal ions and fails to discriminate between essential and non-essential metals. It has also been shown that *zinT* is under the control of a variety of regulators and responds to a wide variety of external stimuli. Therefore, determining the exact role of ZinT in *E. coli* metal ion homeostasis is difficult. However, the data presented here, the homology of ZinT to putative zinc uptake proteins, and the regulation of *zinT* transcription by Zur [18,20], all suggest that ZinT is primarily involved in zinc homeostasis. This study supports the hypothesis that ZinT is a periplasmic zinc chaperone involved in the binding and shuttling of zinc to periplasmic zinc-containing proteins under zinc-limited conditions [20]. The earlier assignment of ZinT (as YodA) as a member of the cadmium stress stimulon may have been due to Zn^{++} ions on Zur being replaced by Cd^{++} at elevated cadmium concentrations, releasing the Zur repressor from its binding site upstream of P*zinT* and resulting in *zinT* expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.09.094.

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