Severe Zinc Depletion of *Escherichia coli*

**ROLES FOR HIGH AFFINITY ZINC BINDING BY ZnT, ZINC TRANSPORT AND ZINC-INDEPENDENT PROTEINS**

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Zinc ions play indispensable roles in biochemical chemistry. However, bacteria have an impressive ability to acquire Zn2+ from the environment, making it exceptionally difficult to achieve Zn2+ deficiency, and so a comprehensive understanding of the importance of Zn2+ has not been attained. Reduction of the Zn2+ content of *Escherichia coli* growth medium to 60 nM or less is reported here for the first time, without recourse to chelators of poor specificity. Cells grown in Zn2+-deficient medium had a reduced growth rate and contained up to five times less cellular Zn2+. To understand global responses to Zn2+ deficiency, microarray analysis was conducted of cells grown under Zn2+-replete and Zn2+-depleted conditions in chemostat cultures. Nine genes were up-regulated more than 2-fold (*p* < 0.05) in cells from Zn2+-deficient chemostats, including *zinT* (*yodA*). *zinT* is shown to be regulated by Zur (zinc uptake regulator). A mutant lacking *zinT* displayed a growth defect and a 3-fold lowered cellular Zn2+ level under Zn2+ limitation. The purified ZnT protein possessed a single, high affinity metal-binding site that can accommodate Zn2+ or Cd2+. A further up-regulated gene, *ykgM*, is believed to encode a non-Zn2+-containing periplasmic zinc-binding protein L31. The gene encoding the enigmatic Zn2+-binding protein *znuA* showed increased expression. During both batch and chemostat growth, cells “found” more Zn2+ than was originally added to the culture, presumably because of leaching from the culture vessel. Zn2+ elimination is shown to be a more precise method of depleting Zn2+ than by using the chelator N,N,N′,N′'-tetrakis(2-pyridylmethyl)ethylenediamine.

Almost all biological interactions depend upon contacts between precisely structured protein domains, and Zn2+ may be used to facilitate correct folding and stabilize the domain (1, 2). Zn2+ also plays an indispensable catalytic role in many proteins (1). Although normally classed as a trace element, Zn2+ accumulates to the same levels as calcium and iron in the *Escherichia coli* cell (3); predicted Zn2+-binding proteins account for 5–6% of the total proteome (4).

However, despite its indispensable role in biology, as with all metals, Zn2+ can become toxic if accumulated to excess. With no subcellular compartments to deposit excess metal, Zn2+ homeostasis in bacteria relies primarily on tightly regulated import and export mechanisms (5). The major inducible high affinity Zn2+ uptake system is the ABC transporter ZnuABC. ZnuA is important for growth (6) and Zn2+ uptake (7) and is thought to pass Zn2+ to ZnuB for transport through the membrane. Zn2+-bound Zur represses transcription of znuABC, whereas the addition of the metal chelator TPEN de-represses expression from a promoterless lacZ gene inserted into znuA, znuB, and znuC (8). Zur can sense subfemtomolar concentrations of cytosolic Zn2+, implying that cellular Zn2+ starvation commences at exceptionally low Zn2+ concentrations (3). O’Hara and O’Halloran (9) found that the minimal Zn2+ content required for growth in *E. coli* is 2 × 10⁵ atoms/cell, which corresponds to a total cellular Zn2+ concentration of 0.2 mM, ~2000 times the Zn2+ concentration found in the medium. A similar cellular concentration of Zn2+ was found in cells grown in LB medium.

Thus, *E. coli* has an impressive ability to acquire and concentrate Zn2+ (3), making the task of depleting this organism of Zn2+ very difficult. Nevertheless, during the course of this work, a paper was published (9) in which the authors conclude that ZnT (formerly YodA) “is involved in periplasmic zinc binding and either the subsequent import or shuttling of zinc to periplasmic zinc-containing proteins under zinc-limiting conditions.” Surprisingly, this conclusion was drawn from experiments in which Zn2+ levels in the medium were lowered only by reducing the amount of Zn2+ added, without metal extraction or chelation.

Only a few attempts have been made to study the global consequences of metal deficiency using “omic” technologies. A study using TPEN (10) found 101 genes to be differentially regulated in *E. coli*. However, the authors note that TPEN has been reported to bind Cd2+, Co2+, Ni2+, and Cu2+ more tightly than it binds Zn2+, and indeed, 34 of the 101 differentially regulated proteins were Cd2+-dependent.

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genes are transcriptionally regulated by Fur (the iron (Fe) uptake regulator) or involved in iron or copper metabolism. Thus, the transcriptome of *E. coli* associated with Zn\(^{2+}\) deficiency alone has not been elucidated. Most genome-wide microarray studies of the effects of metal stresses to date have been carried out in batch culture, but continuous culture offers major benefits for such studies. The greater biological homogeneity of continuous cultures and the ability to control all of the relevant growth conditions, such as pH and especially growth rate, eliminate the masking effects of secondary stresses and afford to Zn\(^{2+}\) deprivation by Zur and that ZinT has a high affinity for Zn\(^{2+}\). We also reveal roles for Zn\(^{2+}\) redistribution in surviving Zn\(^{2+}\) starvation. We demonstrate here for the first time that one such gene, zinT, is up-regulated in response to extreme Zn\(^{2+}\) deprivation by Zur and that ZinT has a high affinity for Zn\(^{2+}\). We also reveal roles for Zn\(^{2+}\) redistribution in surviving Zn\(^{2+}\) deficiency.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Bacterial strains used in this study are listed in Table 1. The cells were grown in glycerol-glycerophosphate medium (GGM), slightly modified from Beard et al. (15). GGM is buffered with MES, which has minimal metal chelating properties, and uses organic phosphate as the phosphate source to minimize formation of insoluble metal phosphates (16). The final concentrations are: MES (1.00 mM), EDTA (134 μM), CaCl\(_2\)\(\cdot\)2H\(_2\)O (68.0 μM), FeCl\(_3\)\(\cdot\)6H\(_2\)O (18.5 μM), ZnO (6.14 μM), H\(_3\)BO\(_3\) (1.62 μM), CuCl\(_2\)\(\cdot\)2H\(_2\)O (587 nm), Co(NO\(_3\))\(_3\)\(\cdot\)6H\(_2\)O (344 nm), and (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\)\(\cdot\)4H\(_2\)O (80.9 nm) in MilliQ water (Millipore). Bulk elements (MES, NH\(_4\)Cl, KCl, K\(_2\)SO\(_4\), and glycerol in MilliQ water at pH 7.4 (batch growth) or 7.6 (continuous culture)) were passed through a column containing Chelex-100 ion exchange resin (Bio-Rad) to remove contaminating cations. Trace elements (with or without Zn\(^{2+}\) as necessary) and a CaCl\(_2\) solution were then added to give the final concentrations shown above prior to autoclaving. After autoclaving, MgCl\(_2\) and β-glycerophosphate were added at the final concentrations shown. All of the chemicals were of AnalAR grade purity or higher. Chelex-100 was packed into a Bio-Rad Glass Econo-column (≈120 × 25 mm) that had previously been soaked in 3.5% nitric acid for 5 days.

**Creating Zn\(^{2+}\)-deficient Conditions and Establishing Zn\(^{2+}\)-limited Cultures**—Culture vessels and medium were depleted of Zn\(^{2+}\) by extensive acid washing of glassware, the use of a chemically defined minimal growth medium, chelation of contaminating cations from this medium using Chelex-100, and the use of newly purchased high purity chemicals and metal-free pipette tips. Plastics that came into contact with the medium (e.g., bottles, tubes, and tubing) were selected on the basis of their composition and propensity for metal leaching, and included polypropylene, polyethylene, polytetrafluoroethylene (PTFE), or polyvinyl chloride. Dedicated weigh boats, spatulas, measuring cylinders, PTFE-coated stir bars, and a pH electrode were used. PTFE face masks, polyethylene gloves, and a PTFE-coated thermometer were also used. The solutions were filter-sterilized using polypropylene syringes with no rubber seal, in conjunction with syringe filters with a PTFE membrane and polypropylene housing. Vent filters contained a PTFE membrane in polypropylene housing. The cells were grown in continuous culture in a chemostat that was constructed entirely of nonmetal parts as detailed below.

**Continuous Culture of E. coli Strain MG1655—E. coli strain MG1655 was grown in custom-built chemostats made entirely of nonmetal parts essentially as described by Lee et al. (14) with some modifications. Glass growth vessels and flow-back traps were soaked extensively (approximately two months) in 10% nitric acid before rinsing thoroughly in MilliQ water. Vent filters (Vent Acro 50 from VWR) were connected to the vessel using PTFE tubing. Metal-free pipette tips were used (MAXYMum Recovery Filter Tips from Axygen). Culture volume was maintained at 120 ml using an overflow weir in the chemostat vessel (14). The vessel was inoculated using one of the side arms. Flasks were stirred on KMO 2 Basic IKA-Werke stirrers at 437 rpm determined using a handheld laser tachometer (Compact Instruments Ltd). The use of a vortex impeller suspended from above the culture avoided grinding of the glass vessel that would occur if a stir bar were used. The samples were taken from the culture vessel as in Lee et al. (14). The dilution rate (and hence the specific growth rate) was 0.1 h\(^{-1}\) (which is below the maximal specific growth rate \(\mu_{\text{max}}\) for this strain (17)). No washout was observed in long term chemostat cultures in Zn\(^{2+}\)-depleted medium. One chemostat was fed medium that contained “adequate” Zn\(^{2+}\) (i.e., normal GGM concentration), whereas the other contained no added Zn\(^{2+}\) and had been depleted of Zn\(^{2+}\) as above. Chemostats were grown for 50 h to allow five culture volumes to pass through the vessel and allow an apparent (pseudo-)steady state to be reached. More prolonged growth was avoided to minimize the
formation of mutations in the rpoS gene (18). Samples were taken throughout to check pH, $A_{600}$, glycerol content and for contaminants. Steady state values for pH and $A_{600}$ were 6.9 and 0.6, respectively. Glycerol assays (19) showed cultures to be glycerol-limited.

The Zn$^{2+}$-free chemostat was inoculated with cells that had been subcultured in Zn$^{2+}$-free medium. A 0.25-ml aliquot of a saturated culture of strain MG1655 grown in LB was centrifuged, and the pellet was used to inoculate 5 ml of GGM that was incubated overnight at 37 °C with shaking. A 2.4-ml (i.e. 2% of chemostat volume) aliquot of this was then used to inoculate the chemostat. The adequate Zn$^{2+}$ chemostat was inoculated with cells treated in essentially the same way but grown in GGM containing adequate Zn$^{2+}$. The two cultures (± Zn$^{2+}$) used to inoculate the chemostats had $A_{600}$ readings within 2.5% of each other. Aliquots from the chemostat were used to harvest RNA and for metal analysis by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; see below).

**Batch Growth of E. coli Strains in GGM ± Zn$^{2+}$** — A saturated culture was grown in LB (with antibiotics as appropriate). To minimize carry-over of broth, the cells were collected from ~0.25 ml of culture by centrifugation, and the pellet was resuspended in a 5-ml GGM starter culture (with Zn$^{2+}$ and antibiotics as appropriate) for 24 h. Side arm flasks containing 25 ml GGM with Zn$^{2+}$ were then inoculated with the equivalent of 1 ml of a culture with $A_{600}$ of 0.6. For these experiments, cultures with zinc were grown in medium containing adequate Zn$^{2+}$ where no special precautions were taken in preparing the medium. Zinc-depleted cultures were grown in side arm flasks that had been soaked extensively in 10% nitric acid before being rinsed thoroughly in MilliQ water. Growth was measured over several hours using a Klett colorimeter and a red filter (number 66; Manostat Corporation). The colorimeter was blanked using GGM. No antibiotics were present in the growth medium used for batch growth curves because they can act as chelators (20–23), but cultures were spotted onto solid LB plates with and without antibiotics at the end of the growth curve to verify that antibiotic resistance was retained. At the end of the growth curve, aliquots of the culture were combined and pelleted for ICP-AES analysis (see below).

**RNA Isolation and Microarray Procedures** — These were conducted as described by Lee et al. (14). RNA was quantified using a BioPhotometer (Eppendorf). *E. coli* K-12 V2 OciChip microarray slides were purchased from Ocmium Biosolutions Ltd. (previously MWG Biotech). Biological experiments (i.e. comparison of low Zn$^{2+}$ versus adequate Zn$^{2+}$ in chemostat culture) were carried out three times, and a dye swap was performed for each experiment, providing two technical repeats for each of the three biological repeats. The data were analyzed as before (14). Spots automatically flagged as bad, negative, or poor in the Imagene software were removed before the statistical analysis was carried out in GeneSight.

**zinT Gene Inactivation** — The zinT gene was functionally inactivated by the insertion of a chloramphenicol resistance cassette using the method of Datsenko and Wanner (24). The pACYC184 chloramphenicol resistance cassette was amplified by PCR using primers that have 40 bases of identity at their 5’ ends to regions within the zinT gene. The forward primer was 5’-GCATGGTCATCACTCACAGGCCAACCCCTTAACAGAGGTCAAGCCACTGGAGCACCTCAA-3’ and the reverse was 5’-CAATGGCGTCTCTCAATGCAAATCTCTCGATATCTTGTTCAGGGGAGGCTGAGCCAA-3’ (the regions homologous to zinT are underlined). The linear DNA was used to transform strain RKP5082 by electroporation. This strain contains pKD46, which overexpresses the phage λ recombination enzymes when arabinose is present. Bacteria were grown to an $A_{600}$ of 0.6 in 500 ml of LB containing ampicillin (final concentration, 150 μg/ml) and arabinose (final concentration, 1 mM) at 30 °C. The cells were then pelleted and made electrocompetent by washing the pellet three times in ice-cold 10% glycerol. The last pellet was not resuspended but vortexed into a slurry. Aliquots of cells (50–100 μl) were electroporated with 1–10% linear DNA (v/v) at 1800 V. The cells were recovered by the addition of 1 ml of LB and incubation at 37 °C for 90 min. The cells were then pelleted and plated onto LB containing chloramphenicol at 34 μg/ml (final concentration). Loss of pKD46 plasmid was checked by streaking transformants on LB agar plates containing ampicillin (final concentration, 150 μg/ml). Insertion of the chloramphenicol cassette was checked by DNA sequencing. The zinT:cam (chloramphenicol resistance cassette) mutant strain was named RKP5456.

**Construction of a λΦ(P<sub>zinT</sub>-lacZ) zur:Sp<sup>e</sup> Strain** — The zur:Sp<sup>e</sup> (spectinomycin resistance cassette) mutation in strain SIP812 (8) was moved into strain AL6, which harbors the λΦ(P<sub>zinT</sub>-lacZ) fusion (25), by P1 transduction (26). The strain was named RKP5475.

**Quantitative Real Time (qRT)-PCR** — This was carried out on RNA samples harvested from the chemostats exactly as described in Lee et al. (14). The mRNA levels of holB were unchanged as determined by array analysis and were thus used as an internal control.

**ICP-AES** — Cells (from 25 ml (batch) or ~85 ml of culture (chemostat)) were harvested by centrifugation at 5000 × g for 5 min (Sigma K15) in polypropylene tubes from Sarstedt (catalogue numbers 62.547.004 (50 ml) or 62.554.001 (15 ml)). Culture supernatants were retained for analysis. The pellets were washed three times in 0.5 ml of 0.5% HNO<sub>3</sub> (Aristar nitric acid, 69% v/v) to remove loosely bound elements. Supernatants collected from the washes were also retained for analysis.

The pellets were resuspended in 0.5 ml of HNO<sub>3</sub> (69%) before transfer to nitric acid-washed test tubes (previously dried). The samples were placed in an ultrasonic bath for ~30 min to break the cells. The resultant digest was then quantitatively transferred to a calibrated 15-ml tube and made up to 5 ml with 1% HNO<sub>3</sub>. The samples were analyzed using a Spectrociros<sup>®</sup>CDD (Spectroanalytical) inductively coupled plasma-atomic emission spectrometer using background correction. Analyte curves were created for each element to be tested using multi-element standard solutions containing 0.1, 0.2, 1, 5, and 10 mg liter<sup>−1</sup>. The wavelengths (nm) for each element were as follows: calcium, 183.801; cobalt, 228.616; copper, 324.754 and 327.396; iron, 259.941; magnesium, 279.079; molybdenum, 203.030; sodium, 589.592; and zinc, 213.856. A 1% nitric acid solution in MilliQ water was used as a blank to and dilute cell digests before ICP-AES analysis. Concentrations of each element in each sam-
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ple (pellets, culture supernatants, and wash supernatants) were calculated using the standard curves. The measurements obtained were the means of five replicate integrations. The limit of Zn\(^{2+}\) detection was 0.001 mg liter\(^{-1}\) (i.e., 1 ppb). In the “simple” low matrix solutions analyzed here, the wavelength used for Zn\(^{2+}\) detection is interference-free and specific for Zn\(^{2+}\).

Elemental recoveries were calculated from these samples. Two different recovery calculations were performed: 1) the percentage of an element in the culture that was subsequently recovered in the washed cell pellet, wash supernatants, and culture supernatant, and 2) the percentage of an element recovered in the unwashed pellet and culture supernatant. The former was used for batch and chemostat samples, and the latter was used for chemostat only. In some samples, element concentrations were below the calculated limit of detection (LOD) for the method. LOD is calculated from the calibration curve based on three \(\sigma\) of a blank signal. Where the signal is at or below the LOD, the instrument reports a < LOD value. In these cases, the LOD is used in subsequent calculations, so it will be an overestimation. Detection of Zn\(^{2+}\) was further complicated because, in many cases, Zn\(^{2+}\) concentrations were close to unavoidable background levels.

Calculation of Dry Cell Weight—Cellular metal contents were expressed on a dry cell mass basis. This was determined by filtering known volumes of culture (10, 20, and 30 ml) through preweighed cellulose nitrate filters (47-mm diameter and pore size of 0.2 \(\mu\)m; Millipore). The filters had previously been dried at 105 °C for 18–24 h to constant weight. The filters were again dried at 105 °C for 18–24 h to constant weight. The pellets were stored at −80 °C for later use; a cell pellet derived from 1 liter of culture was resuspended in ~15 ml of buffer (50 mM Tris/MOPS, 100 mM KCl, pH 8) and sonicated on ice to break the cells. Cell debris was pelleted by centrifugation for 30 min at 12 000 \(\times\) g at 4 °C, whereupon the supernatant was removed and further centrifuged for 15 min at 27 000 \(\times\) g. The cleared lysate was then loaded into a 5-ml TALON resin column, washed with 50 ml of buffer P, followed by 50 ml of buffer P containing 20 mM imidazole. The cleared lysate was then loaded into a 5-ml TALON resin column, washed with 50 ml of buffer P, followed by 50 ml of buffer P containing 20 mM imidazole. Thrombin (60–80 units in 3–4 ml of buffer P) was pipetted onto the column, allowed to soak into the resin, and incubated overnight at room temperature. Ten 1-ml fractions were eluted using buffer P. Recombinant ZnT was determined to be >95% pure by SDS-PAGE. Protein was quantified using its absorbance at 280 nm and the theoretical extinction coefficient of 35995 M\(^{-1}\) cm\(^{-1}\) (estimated using the web-based program ProtParam at ExPASy), which assumes that all cysteines in the protein appear as half-cysteines using information based on (28). The theoretical extinction coefficient is based on the protein sequence minus the periplasmic targeting sequence.

\(N\)-terminal Protein Sequencing—Following SDS-PAGE, purified YodA was blotted onto a polyvinylidene fluoride membrane. The fragment of interest was excised from the membrane, and the sequence was determined using an Applied Biosystems Procise 392 protein sequencer.

Assays of Metal Binding to Purified ZnT—Purified recombinant ZnT was exchanged into buffer D (20 mM MOPS, pH 7) using a PD-10 desalting column (GE Healthcare). ZnT (1 ml) was incubated with various concentrations of ZnSO\(_4\times\)H\(_2\)O (ACS grade reagent) and/or CdCl\(_2\times\)2H\(_2\)O (AnalaR grade) for 1 h at room temperature. The protein/metal mixture was then loaded onto a PD-10 column and eluted in 7 × 0.5-ml fractions using buffer D. The fractions were assayed for A\(_{280}\) and for metal content using ICP-AES. Quantification of some elements was below the LOD in a limited number of samples that do not affect the overall interpretation of the experiment. In these cases the value for the LOD was used for subsequent calculations and thus will be an overestimation.
Mag-fura-2 Binding Experiments—Purified recombinant ZnT was exchanged into buffer (140 mM NaCl, 20 mM Hepes, pH 7.4) using a PD-10 desalting column. Absorption spectra were collected using a Varian Cary 50 Bio UV-visible spectrophotometer at 37 °C. Buffer composition and experimental conditions were taken from Simons (47). ZnT (500 μl; ~15 μM) was placed in a quartz cuvette, and a spectrum was taken from which the concentration of ZnT was determined. Difference spectra were recorded in which the reference sample was buffer M. Equimolar mag-fura-2 (MF; Molecular Probes, catalogue number M-1290) was then added. Aliquots of ZnSO₄·7H₂O (ACS grade reagent) and/or CdCl₂·2H₂O (AnalAR grade) in buffer M were added, mixed, and incubated for 1 min before the spectra were collected. The equilibrium was established within 1 min of Zn²⁺ being added.

**RESULTS**

Creating Zn²⁺-deficient Conditions—Several precautions, based on normal analytical practice and the findings of Kay (29) regarding Zn²⁺ contamination, were taken to ensure that culture vessels and medium were depleted of Zn²⁺ where necessary. Table 2 shows typical values for the amounts of various metals in GGM as analyzed by ICP-AES. Both Zn²⁺-depleted and -replete media showed good correlation with the expected values. In various batches of media analyzed, Zn²⁺ concentrations in Zn²⁺-depleted medium ranged from <0.001 to 0.004 mg liter⁻¹ (<15–60 nM Zn²⁺). The variation in Zn²⁺ depletion achieved is a result of the difficulty in excluding Zn²⁺ from all sources that come into contact with the medium and culture. Sodium was used as the exchanging ion on Chelex-100, but excess sodium was not detected in the medium following chelexation (data not shown).

Growth in Zn²⁺-depleted Batch Cultures—E. coli strain MG1655 was grown in GGM with or without Zn²⁺ (Fig. 1A). The Zn²⁺-limited culture showed a lag in entering the exponential phase, and a semi-logarithmic analysis of growth (not shown) revealed that the Zn²⁺-limited culture had an increased doubling time (159.0 min) compared with the Zn²⁺-replete culture (125.4 min) and reached a lower final A value. Because A measurements may reflect cell size changes (30), the samples were taken at the end of growth for electron microscopy, but no discernible size difference was seen between E. coli cells grown with or without Zn²⁺ in GGM (not shown). Cells grown in GGM (±Zn²⁺) were, however, smaller (length, width, and volume) than cells grown in rich medium (LB), presumably because of a slower growth rate (31).

**TABLE 2**

Expected and representative measured amounts of elements in Zn²⁺-sufficient and -depleted GGM

| Element       | Predicted from medium composition | Measured by ICP-AES |
|---------------|----------------------------------|---------------------|
|               | mg liter⁻¹                       | Zn²⁺-sufficient     | Zn²⁺-depleted |
| Zinc          | 0.401/0                          | 0.340               | 0.004        |
| Iron          | 1.945                            | 0.886               | 0.878        |
| Copper        | 0.037                            | 0.033               | 0.034        |
| Cobalt        | 0.0257                           | 0.018               | 0.019        |
| Molybdenum    | 0.054                            | 0.068               | 0.059        |
| Calcium       | 2.28                             | 2.83                | 2.85         |
| Magnesium     | 24.0                             | 24.2                | 25.0         |

GGM contains EDTA, which prevents precipitation of the trace elements present. This is a well established and common practice (17). However, to investigate whether this EDTA was itself creating Zn²⁺ depletion, we cultured MG1655 in GGM with and without EDTA (supplemental Fig. S1). When grown in GGM without EDTA, MG1655 displayed a longer lag phase and reduced growth yield. The growth rate was also affected; the doubling time during exponential growth increased from 125.5 (with EDTA) to 131.5 min (without EDTA). Thus, EDTA is not creating a state of Zn²⁺ depletion but rather is a beneficial component of the medium.

As well as growing at a reduced rate, cells grown in Zn²⁺-depleted medium had ~1.8–5.0-fold less cellular Zn²⁺ than those grown in Zn²⁺-replete medium (based on three separate experiments). For example, at the end of the growth curve shown in Fig. 1A, the cells cultured in Zn²⁺-replete medium contained 1.12 × 10⁻⁵ mg of Zn²⁺/mg of dry weight cells and the cells grown in Zn²⁺-depleted medium contained 3.40 × 10⁻⁶ mg of Zn²⁺/mg of dry weight cells (a 3.3-fold difference). Here, ”cellular Zn” is defined as that which cannot be removed by three successive washes with 0.5% nitric acid. To verify the reliability of the metal analyses, elemental recoveries were calculated from these samples. Fig. 2 shows that, for cells grown in Zn²⁺-replete medium, Zn²⁺ recovery was between 90 and 110%, and for cells grown in Zn²⁺-replete and Zn²⁺-deplete medium, the recovery of iron, copper, cobalt, and magnesium was also between 90 and 110%. For these elements, therefore, the metal content in the washed pellet and the culture supernatant and the wash supernatants fully accounts for the metal initially added to the culture in the medium. However, this was not true for Zn²⁺ recovery in cells grown in Zn²⁺-deficient medium. Zn²⁺ in these cells, together with that in the culture supernatant and wash supernatants, was 5-fold higher than the amount originally added to the culture in the medium. This
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suggests an avid Zn\(^{2+}\) sequestering ability of cells cultured under limiting Zn\(^{2+}\) conditions. Details of the analyses of individual pellets, wash solutions, supernatants, and media for Zn\(^{2+}\) are found in supplemental Table S1. We conclude that Zn\(^{2+}\) limitation can be achieved in batch culture without resorting to chelators despite effective bacterial Zn\(^{2+}\) scavenging mechanisms.

**Cells Grown in Continuous Culture “Find” Extra Zn\(^{2+}\)—**To explore Zn\(^{2+}\) acquisition and localization at constant growth rates and defined conditions for a detailed transcriptomic study, *E. coli* strain MG1655 was grown in parallel glycerol-limited chemostats, one fed with medium that contained “adequate” Zn\(^{2+}\) and one that had been rigorously depleted of Zn\(^{2+}\). For the majority of elements assayed (iron, copper, cobalt, magnesium, molybdenum, potassium, sodium, phosphorus, and sulfur), the percentage recoveries were 90–110% (data not shown). However, more Zn\(^{2+}\) was recovered from the cells grown in the Zn\(^{2+}\)-deficient chemostat than was originally added to the culture (Table 3), as in batch culture (Fig. 2). This is presumed to be due to active leaching from glassware or carry-over from the inoculum. Interestingly, this percentage markedly decreased with successive experiments in the same chemostat apparatus, suggesting that there is less Zn\(^{2+}\) able to be leached after repeated runs of culture in the same chemostat vessel (Table 3). Details of the analyses of individual pellets, wash solutions, supernatants, and media are found in supplemental Table S2.

Cells grown in the Zn\(^{2+}\)-deficient chemostat consistently contained less cellular Zn\(^{2+}\) than those grown in Zn\(^{2+}\)-replete medium (e.g. \(2.94 \times 10^{-5}\) mg of Zn\(^{2+}\)/mg of cells grown grown in adequate Zn\(^{2+}\) and \(0.536 \times 10^{-5}\) mg of Zn\(^{2+}\)/mg of cells for cells harvested from run 5 of the Zn\(^{2+}\)-limited chemostat (a 5.5-fold decrease)).

**Transcriptome Changes Induced by Zn\(^{2+}\) Deficiency—**The genome-wide mRNA changes of strain MG1655 grown in continuous culture with adequate or limiting Zn\(^{2+}\) were probed using microarray technology. Commonly applied criteria to determine the significance in transcriptomic studies are a fold change of more than 2 and a \(p\) value of less than 0.05. Using these criteria, of the 4288 genes arrayed, only nine showed significant changes (an increase in all cases) in mRNA levels and are listed in Table 4. Genes not meeting these criteria may be biologically significant but are not studied further here. It should be noted that microarrays measure the relative abundance of mRNA but cannot inform as to whether changes occur because of changes in the rate of transcription or because of changes in the stability of the transcript. Zn\(^{2+}\) has been reported to affect the stability of the mRNA of a human Zn\(^{2+}\) transporter (32). The full data set has been deposited in GEO (accession number GSE11894) (33). Three genes were chosen for further study based on known links to Zn\(^{2+}\) homeostasis. The remaining six genes were not studied further. In total, 21 genes displayed a greater than 2-fold increase in mRNA levels,

### TABLE 3

| Run | Washed cell pellet + wash solutions | Unwashed cell pellet + supernatant |
|-----|----------------------------------|----------------------------------|
|     | +Zinc | −Zinc | +Zinc | −Zinc |
| 1   | 104   | 1858  | ND    | ND    |
| 2   | 110   | 1676  | ND    | ND    |
| 3   | 105   | 559   | ND    | ND    |
| 4   | 104   | 493   | 102   | 454   |
| 5   | 103   | 248   | 103   | 254   |

### TABLE 4

| Gene | b number | Gene product | Fold increase | \(p\) value (<0.05) |
|------|----------|--------------|---------------|---------------------|
| *zinT* | b1973 | Periplasmic cadmium-binding protein; induced by cadmium and peroxide; binds zinc, nickel, and cadmium; SoxS- and Fur-regulated | 8.07 | 0.0001 |
| *znuA* | b1857 | High affinity ABC transport system for zinc, periplasmic | 2.88 | 0.00117 |
| *fdrG* | b1474 | Formate dehydrogenase-N, selenopeptide, anaerobic; periplasmic | 2.86 | 0.00386 |
| *emtA* | b1193 | Membrane-bound transglycosylase E, lipoprotein; involved in limited murein hydrolysis | 2.86 | 0.00998 |
| *ykgM* | b0296 | RpmE paralogue, function unknown | 2.64 | 0.03647 |
| *mdtB* | b2077 | Putative transporter, function unknown; no MDR phenotype when mutated or cloned; fourth gene in *mdtABCD*BAES operon | 2.46 | 0.01614 |
| *ribA* | b1277 | GTP cyclohydrolase II, riboflavin biosynthesis | 2.36 | 0.02506 |
| *yaeE* | b1577 | Pseudogene, N-terminal fragment, Qtn prophage | 2.17 | 0.00452 |
| *aslA* | b3801 | Suppresses gsp mutants; putative arylsulfatase | 2.15 | 0.02660 |
Hypersensitivity of Selected Strains to Zn\(^{2+}\) Deficiency

The results obtained by microarray experiments, several genes that were induced by Zn\(^{2+}\) depletion were examined by qRT-PCR to determine independently relative mRNA levels. The levels of up-regulation determined by qRT-PCR (mean ± normalized standard deviation) were as follows: yodA, 7.77 ± 0.63; ykgM, 2.83 ± 0.61; and zurA, 2.34 ± 0.58. These values correspond closely to increases in the microarray analysis of 8.07- to 2.64-, and 2.88-fold, respectively. Similar qRT-PCR values were obtained on one (ykgM and zuuA) or two (yodA) other occasions. The mRNA levels of holB (internal control) were unchanged as determined by qRT-PCR and array analysis.

### Hypersensitivity of Selected Strains to Zn\(^{2+}\) Deficiency

To assess the importance of the ykgM, zinT, and zuuA genes in surviving Zn\(^{2+}\) deficiency, mutants were used in which each gene are inactivated by insertion of an antibiotic resistance cassette; the growth of these isogenic strains was compared in Zn\(^{2+}\)-depleted and Zn\(^{2+}\) replete liquid cultures (Fig. 1). Each strain (wild type and mutants) grew more poorly in the absence of Zn\(^{2+}\) than in its presence. Also, in Zn\(^{2+}\)-depleted medium, the ykgM::kan (kanamycin resistance cassette), zinT::cam, and zuuA::kan mutants consistently grew more poorly than MG1655 in the same medium. We were unable to culture the zuuA::kan mutant to >5 Klett units in the severely Zn\(^{2+}\)-depleted conditions achieved here (Fig. 1D). All of the experi-

### TABLE 5

| Gene    | b number | Gene product                                                                 | Fold change | p value |
|---------|----------|-------------------------------------------------------------------------------|-------------|---------|
| yodB    | b1974    | Function unknown                                                             | 2.38        | 0.0725  |
| zur     | b4046    | Repressor for zuuABC, the zinc high affinity transport genes; dimer; binds two Zn(II) ions per monomer | 1.37        | 0.9578  |
| znuC    | b1858    | High affinity ABC transport system for zinc                                 | 1.36        | 0.2294  |
| znuB    | b1859    | High affinity ABC transport system for zinc                                  | 1.34        | 0.0857  |
| zntA    | b3292    | Zinc-responsive activator of zntA transcription                              | 1.32        | 0.1109  |
| zitB    | b3469    | Zinc-binding periplasmic protein; responsive to Zn\(^{2+}\) and Pb\(^{2+}\); autoregulated; regulation of Hyd-3 activity is probably due to cross-talk of overexpressed protein | 1.25        | 0.9322  |
| zraP    | b4002    | Zinc-binding periplasmic protein; responsive to Zn\(^{2+}\) and Pb\(^{2+}\); autoregulated; regulation of Hyd-3 activity is probably due to cross-talk of overexpressed protein | 1.25        | 0.9322  |
| ydiP    | b3915    | Iron and zinc efflux membrane transporter; cation diffusion                   | 1.17        | 0.2742  |
| zitB    | b6752    | Zn(II) efflux transporter; zinc-inducible                                     | 1.09        | 0.9571  |
| zntA    | b3469    | Zn(II), Cd(II), and Pb(II) translocating P-type ATPase; mutant is hypersensitive to Zn\(^{2+}\) and Cd\(^{2+}\) salts | 1.07        | 0.9285  |
| spy     | b1743    | Periplasmic protein induced by zinc and envelope stress, part of cpxR and haeSR regulon | 1.03        | 0.8314  |
| zraR    | b4004    | Two component response regulator for zraP; responsive to Zn\(^{2+}\) and Pb\(^{2+}\); autoregulated; regulation of Hyd-3 activity is probably due to cross-talk of overexpressed protein | 0.95        | 0.9315  |
| zupT    | b3040    | Zinc and other divalent cation uptake transporter                            | 0.88        | 0.3258  |

* Insufficient data available to obtain a p value.
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![Graph](image)

FIGURE 3. β-Galactosidase activity of λP_<sub>zinT</sub>-lacZ under various conditions. A and B, β-galactosidase activity of λP_<sub>zinT</sub>-lacZ (strain AL6) grown in GGM containing the concentrations of Zn<sup>2+</sup>, Cd<sup>2+</sup>, and TPEN shown. The Zn<sup>2+</sup> concentrations can be interpreted as follows: 6.14 μM is GGM in which the bulk elements were Chelex-100-treated and then trace elements containing Zn<sup>2+</sup> were added back; <0.06 μM is GGM in which extreme precautions were taken to exclude Zn<sup>2+</sup> (see text). The cultures were harvested when the A<sub>600</sub> reached 0.2–0.4. The means ± standard deviation for three technical replicates are shown. The same results were seen on at least one other occasion. C and D, β-galactosidase activity of λP_<sub>zinT</sub>-lacZ in a zur::Sp<sup>c</sup> background (strain RKP5475) grown in GGM containing the Zn<sup>2+</sup> and TPEN concentrations shown. The cultures were harvested when the A<sub>600</sub> reached 0.2–0.4. The means and standard deviations of three technical replicates are shown. The means were carried out in triplicate, and similar results were seen on at least two separate occasions. We confirmed by qRT-PCR that the genes downstream of ykgO, zinT, and zurA (i.e., ykgO, yodB and yebA, respectively) were in all cases transcribed in the mutant strains.

We measured cellular Zn<sup>2+</sup> levels in bacteria grown in conditions of severe Zn<sup>2+</sup> limitation in batch culture. The levels of Zn<sup>2+</sup> detected in cell digests on analysis by ICP-AES were exceedingly low. Nevertheless, the zinT::cam strain contained ~9-fold less cellular Zn<sup>2+</sup> when cultured under Zn<sup>2+</sup> limitation (1.28 × 10<sup>-6</sup> mg of Zn<sup>2+</sup>/mg of cells) than when grown in Zn<sup>2+</sup>-replete (1.16 × 10<sup>-5</sup> mg of Zn<sup>2+</sup>/mg of cells) conditions. Also, under Zn<sup>2+</sup>-deficient conditions, the zinT::cam strain contained nearly 3-fold less cellular Zn<sup>2+</sup> than MG1655 wild-type cells grown under similar conditions (1.28 × 10<sup>-6</sup> mg of Zn<sup>2+</sup>/mg of cells and 3.40 × 10<sup>-6</sup> mg of Zn<sup>2+</sup>/mg of cells, respectively). These data are the first to demonstrate a role for ZinT in Zn<sup>2+</sup> acquisition under strictly Zn<sup>2+</sup>-limited conditions. When the znuA::kan mutant was assayed after growth in Zn<sup>2+</sup>-depleted conditions, the measurement of cellular Zn<sup>2+</sup> was below the LOD. Similar results were seen on at least one other occasion.

Transcriptional Regulation of zinT under Various Zn<sup>2+</sup> Concentrations—Having established that zinT transcription was elevated on Zn<sup>2+</sup> depletion, a P_<sub>zinT</sub>-lacZ transcriptional fusion (25), in which lacZ is transcribed from the zinT promoter, was used to investigate an alternative Zn<sup>2+</sup> removal method and the effects of added Cd<sup>2+</sup> and Zn<sup>2+</sup>. Fig. 3A shows that λP_<sub>zinT</sub>-lacZ activity was highly up-regulated under the Zn<sup>2+</sup>-deficient conditions created here (in which Zn<sup>2+</sup> is excluded from the medium). These data were compared with cultures treated with TPEN (Fig. 3B), which is widely used as a Zn<sup>2+</sup> chelator (3, 7, 32, 42, 43, 45). Fig. 3B shows that expression from λP_<sub>zinT</sub>-lacZ was higher in cells grown in medium containing TPEN than in cells grown in adequate Zn<sup>2+</sup> it was lower than that of cells grown in medium from which Zn<sup>2+</sup> has been rigorously eliminated (Fig. 3A). In LB medium, the P_<sub>zinT</sub>-lacZ fusion strain has previously been shown to respond to elevated levels of Cd<sup>2+</sup> but not of Zn<sup>2+</sup> (25). In GGM, the construct was again unresponsive to elevated Zn<sup>2+</sup>, but no response was seen to elevated Cd<sup>2+</sup> (Fig. 3A), although this may be due to difficulties in growing cells at high levels of Cd<sup>2+</sup>, which were near its maximum permissive concentration.

A Zur-binding site has been reported in the zinT promoter (41), and Zn<sup>2+</sup>-bound Zur represses the transcription of znu-ABC (8). Therefore, to test the hypothesis that Zur also negatively regulates zinT, λP_<sub>zinT</sub>-lacZ activity was monitored in a strain lacking zur. Fig. 3 (C and D) shows that, in a zur mutant,
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\( \lambda \Phi (p_{\text{zinT-lacZ}}) \) activity was not dependent on the extracellular Zn\(^{2+}\) concentration under any condition tested. Thus, Zur is a negative regulator of \( \text{zinT} \) transcription.

**Stoichiometric Binding of Zn\(^{2+}\) and Cd\(^{2+}\) by ZinT**—To investigate the possible role of ZinT in metal binding as suggested by the transcription and growth studies reported here, the \( \text{zinT} \) gene was cloned into pET28a such that the translated protein lacked the periplasmic signal sequence but was fused to a polyhistidine tag and thrombin cleavage site to aid purification. The polyhistidine tag was removed by cleavage with thrombin to minimize the danger of the protein adopting aberrant conformations. The sequence of the resultant protein, which was used to calculate the extinction coefficient, mimics the form of the protein found in the periplasm. Residual imidazole in the final ZinT preparation was avoided by using only a single wash step containing imidazole (20 mM) during purification and exchange into a buffer lacking imidazole before final use. The effective removal of the polyhistidine tag was confirmed by N-terminal sequencing. The pure recombinant protein (Fig. 4A) was incubated with different molar ratios of Zn\(^{2+}\) and then subjected to size exclusion chromatography to assess so the experiment was also carried out using Cd\(^{2+}\). ZinT co-elutes from a size exclusion column with up to 1 molar equivalent of Cd\(^{2+}\), even when initially incubated with more (Fig. 5, A–D). When 13.3 nmol of ZinT was incubated without Cd\(^{2+}\) prior to size exclusion chromatography, the eluate contained less than 18 pmol of Cd\(^{2+}\)/fraction (not shown). It should be noted that, in the case of Cd\(^{2+}\), the Cd\(^{2+}\)/ZinT ratio was 0.9 but never exceeded 1 (Fig. 5D) unlike the case with Zn\(^{2+}\) (Fig. 4F). This is attributable to the inevitable contamination of reagents and materials with Zn\(^{2+}\) but not Cd\(^{2+}\).

To investigate competition of Zn\(^{2+}\) and Cd\(^{2+}\) for site(s) in ZinT, the protein was incubated with both metals, and co-elution of Zn\(^{2+}\) with ZinT. Fig. 4 shows the elution profiles of ZinT and Zn\(^{2+}\) following incubation of ZinT with 0, 0.25, 0.5, 1, and 2 molar equivalents of Zn\(^{2+}\). Fig. 4B (and Fig. 5, A–D) shows that, even when no Zn\(^{2+}\) is added, ZinT co-eluted from the size exclusion column with Zn\(^{2+}\). The occupancy of Zn\(^{2+}\) observed under these conditions (0.6 mol of Zn\(^{2+}\)/mol of ZinT) was approximately half that observed at superstoichiometric Zn\(^{2+}\)/ZinT ratios (Fig. 4F), and so we conclude that the Zn\(^{2+}\) content shown in Fig. 4B represents \( \sim 0.5 \) Zn\(^{2+}\)/ZinT. This suggests a high affinity of ZinT for Zn\(^{2+}\) and is reminiscent of the crystallization of ZinT (38); crystals formed in the absence of added metals contained Zn\(^{2+}\) or Ni\(^{2+}\), indicative of high metal affinity (see “Discussion”). When ZinT was incubated with 0.25 or 0.5 molar equivalents of Zn\(^{2+}\) (Fig. 4, C and D) more Zn\(^{2+}\) co-eluted with ZinT than was originally added. However, when 1 (Fig. 4E), 2 (Fig. 4F), or 3 (data not shown) molar equivalents Zn\(^{2+}\) were incubated with ZinT, approximately one equivalent eluted from the column with the protein. These data provide evidence that ZinT binds one Zn\(^{2+}\) ion with high affinity.

Previous work (38) has suggested that ZinT is able to bind Cd\(^{2+}\), and
Competitive Metal Binding—To estimate the affinity of ZinT for Zn$^{2+}$, Mag-fura-2, a chromophore that binds Zn$^{2+}$ in a 1:1 ratio (46) and with a $K_d$ of 20 nM (47), was used. Its absorption maximum shifts from 366 to 325 nm on Zn$^{2+}$ binding, which is accompanied by a decrease in its extinction coefficient from 29,900 M$^{-1}$ cm$^{-1}$ (MF) to 1880 M$^{-1}$ cm$^{-1}$ (Zn$^{2+}$-MF) (46). Therefore Zn$^{2+}$ binding to MF can be tracked by examining the absorbance at 366 nm (Fig. 6A). Fig. 6B shows a titration of a 1:1 ZinT:MF mixture (filled circles) and MF alone (open circles) with Zn$^{2+}$. When ZinT was not present, the absorbance decreased to zero when 1 molar equivalent of Zn$^{2+}$ had been added. When ZinT was present, however, incremental additions of Zn$^{2+}$ gave smaller decreases in MF absorbance reaching a plateau at 2 molar equivalents of Zn$^{2+}$. This provides good evidence that, although the affinity of ZinT for Zn$^{2+}$ is not high enough to completely outstrip MF of Zn$^{2+}$, ZinT competes with MF for binding of Zn$^{2+}$. The

**FIGURE 5.** Elution profiles of ZinT, Zn$^{2+}$, and Cd$^{2+}$ from a PD-10 column following incubation of protein and metal ions. A–D, elution following incubation of 17.8 nmol of ZinT with 0.5, 1, 2, or 3 molar equivalents of Cd$^{2+}$. Filled circles with solid line, ZinT; open circles with dashed line, Zn$^{2+}$; open triangles with dotted line, Cd$^{2+}$. E and F, elution following incubation of 13.3 nmol of ZinT with 1 molar equivalent of Zn$^{2+}$ and 1 molar equivalent of Cd$^{2+}$ or with 1 molar equivalent of Zn$^{2+}$ and two molar equivalents of Cd$^{2+}$. Filled circles with solid line, ZinT; open circles with dashed line, Zn$^{2+}$; open diamonds with dotted and dashed line, Co$^{2+}$; open triangles with dotted line, Cd$^{2+}$.

**FIGURE 6.** Titration of ZinT and/or MF with Zn$^{2+}$ and/or Cd$^{2+}$. A, representative difference spectra (i.e. minus the protein-only spectrum) of a titration of 14.5 $\mu$M ZinT and 14.5 $\mu$M MF with Zn$^{2+}$ (0.25–3.5 molar equivalents Zn$^{2+}$ in 0.25 steps and then 4–6 molar equivalents in 0.5 steps). The arrows indicate the direction of absorbance changes as Zn$^{2+}$ is added. B, titration of 14.5 $\mu$M ZinT and 14.5 $\mu$M MF with Zn$^{2+}$. C, titration of 14.3 $\mu$M ZinT and 14.3 $\mu$M MF with 1 molar equivalent of Cd$^{2+}$, then Zn$^{2+}$ in 0.5 molar equivalent steps to 4 molar equivalents and then Zn$^{2+}$ in 0.5 molar equivalent steps to 6 molar equivalents. D, titration of 14.1 $\mu$M ZinT and 14.1 $\mu$M MF with 2 molar equivalents of Cd$^{2+}$ and then Zn$^{2+}$ in 0.5 molar equivalent steps. In B–D, absorbance change at 366 nm is plotted against molar equivalents of metal added. The filled circles are in the presence of ZinT; open circles are in the absence of ZinT (MF and buffer only). The lines indicate whether the added metal was Zn$^{2+}$ or Cd$^{2+}$.
For the first time, we have grown Zn²⁺-depleted medium is successfully prepared by eliminating Zn²⁺ during medium preparation and culture. In contrast, chelators can be unspecific, strip metals from exposed sites and increase the availability of certain metals (16). The major disadvantage of using chelators is that the metal is still present in the medium to be picked up by proteins with a higher affinity for the metal than that exhibited by the chelator. For example, ZnuA is able to compete with EDTA for Zn²⁺ (6). Fig. 3A highlights the disadvantage of using chelators to study Zn²⁺ deficiency; the widely used chelator TPEN was less effective than Zn²⁺ elimination, as judged by λΦ(P₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅portion of the document. The text seems to be discussing biological and chemical processes, particularly focusing on metal ion concentrations and their effects on cells. The discussion mentions the ability of Zn²⁺ to compete with other metals such as Cd²⁺, and the role of proteins like ZinT in metal ion homeostasis. The text also highlights the use of chelators like TPEN and their limitations in studying metal ion deficiencies. The discussion is interspersed with references to specific studies and experiments, indicating a research context. The overall theme is the investigation of metal ion homeostasis and the implications of metal ion depletion on cellular processes.
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unusual distorted tetrahedral geometry. Fig. 5 (E and F) shows that, in our hands, ~0.5 molar equivalents Co²⁺ co-elute with the ZinT protein. It is likely that this Co²⁺ has been picked up from the TALON column used during purification, again providing evidence for a high affinity metal-binding site within ZinT. No Ni²⁺ was found in eluting samples (data not shown).

On the basis of the crystallography, David et al. (38) could not conclude which metal would bind to ZinT under physiological conditions. The present study shows clearly that ZinT binds both Zn²⁺ and Cd²⁺ with high affinity. The direct binding experiments (Fig. 5, E and F) show that more Zn²⁺ remains bound to ZinT after size exclusion chromatography than Cd²⁺, providing evidence that Zn²⁺ binds to ZinT more tightly than Cd²⁺. Also, the $K_d$ of MF for Cd²⁺ is greater than for Zn²⁺, so somewhat weaker binding by Cd²⁺ would not be detected in the Mag-Fura-2 competition experiments. Fig. 5 (E and F) shows that more than 1 molar equivalent of metal can bind to the protein. This is consistent with the crystal structure proposed by David et al. (38), which suggests that at least two Zn²⁺ ions can bind in the vicinity of the high affinity site, and that there is additional capacity for further Zn²⁺, up to 4, although this may be due to intermolecular contacts formed during crystallization. The finding that one Zn²⁺ ion is needed to saturate the protein, as assessed by competition with Mag-Fura-2, is entirely consistent with the crystallographic data because this experiment can only report on metal binding to ZinT that is tighter than 20 nM. Although this site in ZinT accommodates different metal ions, the marked accumulation of zinT mRNA by extreme Zn²⁺ limitation strongly suggests that the physiological role of ZinT is ferrying Zn²⁺ ions in the periplasm. Indeed, David et al. (38) suggested that the binding of a second metal, possibly at a lower affinity site, could trigger a conformational change that promotes transport across the membrane or interaction with an unidentified ABC-type transporter. In support of this is the fact that ZinT shows sequence similarity to a number of periplasmic metal-binding receptors of ABC metal transport systems that have been shown to bind Zn²⁺.

In a recent paper (9), growth in media with various Zn²⁺ supplements, or none, was purported to show “dependence of transcriptional response to Zn²⁺ within the ribosome. Upon Zn²⁺ depletion, the $ykgM$-encoded L31 protein is expressed (probably de-repressed by Zur) and becomes preferentially bound to the ribosome (the exact mechanism is unclear), allowing Zn²⁺ within the rpmE-encoded L31 to be recycled. The physiological role of ZinT remains to be fully established, but it may function as a Zn²⁺ chaperone to the membrane-bound Zn²⁺ importer ZnuBC (or a different importer) or mediate direct transport from the periplasm to the cytoplasm. Zn²⁺ is the metal that binds most tightly. This study provides a new appreciation of the regulation of zinT and the role of ZinT in protecting cells from Zn²⁺ depletion.

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