Published in final edited form as:

J Inorg Biochem. 2012 June ; 111: 164–172. doi:10.1016/j.jinorgbio.2011.11.022.

Characterization of Zn(II)-responsive ribosomal proteins YkgM and L31 in *E. coli*

M. Patrick Hensley, Thusitha S. Gunasekera, J. Allen Easton, Tara K. Sigdel, Stacy A. Sugarbaker, Lindsey Klingbeil, Robert M. Breece, David L. Tierney, and Michael W. Crowder*

Department of Chemistry and Biochemistry, 160 Hughes Hall, Miami University, Oxford, OH 45056

Abstract

RT-PCR and DNA microarrays were used to probe for Zn(II)-responsive genes in *E. coli* cells that were made Zn(II) deficient. Microarray data revealed 114 genes were significantly up-regulated and 146 genes were significantly down-regulated in Zn(II) deficient conditions. The three most up-regulated genes were (1) *znuA*, which encodes for a periplasmic protein known to be involved with Zn(II) import, (2) *yodA*, which encodes for a periplasmic protein with unknown function, and (3) *ykgM*, which encodes for a ribosomal protein that is thought to be a paralog of ribosomal protein L31. YodA was over-expressed and purified as a maltose binding protein (MBP) fusion protein and shown to tightly bind 4 equivalents of Zn(II). Metal analyses showed that MBP-YkgM does not bind Zn(II). On the other hand, MBP-L31 tightly binds 1 equivalent of Zn(II). EXAFS studies on MBP-L31 suggest a ligand field of 1 histidine, 1 cysteine, and 2 additional N/O scatterers. Site-directed mutagenesis studies suggest that Cys16 coordinates Zn(II) in MBP-L31 and that the other three cysteines do not bind metal. These results are discussed in light of Zn(II) starvation model that has been postulated for *B. subtilis*.

Keywords

ribosomal protein L31; EXAFS; Zn(II) homeostasis; DNA microarrays

1. Introduction

Zinc is an essential transition metal required for life in all organisms [1]. It plays a key catalytic or structural role in members from all six major classes of enzymes [2–4]. Despite how critical its presence inside cells appears to be, little is understood about the intracellular homeostasis of this metal. Currently, there are two known import systems and three known export systems for Zn(II) in prokaryotic cells. ZntA, ZitB, and YiiP are efflux pumps that remove Zn(II) from the cytoplasm of *E. coli* cells [5, 6]. The transcription of *zntA* is regulated by ZntR, a MerR homolog that up-regulates transcription as a result of Zn(II) binding [7, 8]. ZitB and YiiP are members of the cation diffusion facilitator family that is reported to be regulated by Zn(II) [6, 9]. The ZnuABC and ZupT systems are used to control

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author: Michael W. Crowder, Phone: (513) 529-7274, Fax: (513) 529-5715, crowdemw@muohio.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

influx of Zn(II) into the cytoplasm of *E. coli* cells [10]. The *znuA* operon is transcriptionally-controlled by Zur, a Zn(II)-sensitive Fur homolog [10]. ZupT is a member of the ZIP family of proteins [11]. There is also some evidence that PitA, a phosphate transporter, is zinc responsive and may be involved in zinc transport [12].

In an effort to better understand zinc trafficking, several groups have reported proteomic and cDNA microarray experiments to study the effects of zinc excess and zinc deficiency on *E. coli* [13, 14, 15, 16–18] and to identify Zn(II)-responsive proteins/genes. The rationale for most of these studies is that the expression levels of genes/proteins involved in Zn(II) homeostasis would be affected by cytoplasmic Zn(II) levels. When comparing previous genomic/proteomic experiments however, major differences in growth media and stress times are evident. Poole and coworkers used glycerophosphate-containing minimal medium [14, 15], while Yamamoto *et al.* and Brocklehurst *et al.* used Luria-Bertani medium [13, 16] to study Zn(II)-excess conditions. Sigdel *et al.* used a chemically-defined minimal medium to study Zn(II)-deficient conditions [17]. Stress times varied from 5 minutes by Yamamoto *et al.* [16] to overnight batch cultures by Brocklehurst *et al.* [13], with 5 hours by Sigdel *et al.* [17]. All of these differences have resulted in many differentially-expressed genes being reported, and there is little or no overlap of genes found in the studies. It has recently been shown that the proteomic profile of Zn(II)-stressed *E. coli* cells changes over time [18]. It is unquestionable that gene expression is also time-dependent and that some genes possibly involved in Zn(II) homeostasis were missed due to the incorrect choice of time to harvest the cells after stress for microarray studies.

In *B. subtilis* ribosomal protein L31 and its homolog protein YtiA have recently been hypothesized to participate in a Zn(II) starvation model [19]. Under Zn(II) limiting conditions, the Zn(II)-binding ribosomal protein L31 (called C+ because it is thought to bind Zn(II) with at least two cysteine residues) is replaced with the non Zn(II)-binding paralog YtiA (called C– that lacks the CXXC motif found in L31) [19, 20]. The replacement of Zn(II)-binding L31 with YtiA, which does not bind Zn(II), is thought to mobilize ribosome-associated Zn(II) by releasing Zn(II)-binding ribosomal proteins into the cell that are then degraded and thereby releasing Zn(II). In this model a significant amount of Zn(II) is released into the cell, and ribosome function is maintained. This Zn(II) starvation model suggests that the ribosome may serve as a possible active participant in intracellular Zn(II) homeostasis.

This study was conducted to determine the optimal time for Zn(II)-induced transcription in an effort to identify Zn(II)-responsive genes and if evidence could be found for this Zn(II) starvation model in *E. coli*. Using quantitative, real-time polymerase chain reaction (qPCR), the time-dependent expression levels of most genes predicted to be involved with Zn(II) homeostasis were monitored. It is shown here that several Zur-regulated genes (*znuA*, *ykgM*, *yodA*) were up-regulated as much as 1000-fold over control after 30 minutes of stress, while the levels of other, non *zur*-regulated genes (*zupT*, *zntA*, *zitB*) as well as 16S RNA did not change. The optimum time of stress was used to refine and enhance the subsequent cDNA array studies [17], which revealed 114 genes up-regulated and 146 down-regulated. All of the Zur-regulated proteins, with genes on the microarray chips, were over-expressed, purified, and analyzed for Zn(II) binding. The *ykgM* gene, which displayed the largest up-regulation, encodes a hypothetical protein that is a homolog of the Zn(II)-binding, ribosomal protein L31 [21]. YkgM and L31 were cloned, over-expressed, purified, and subjected to elemental analyses. The data show that L31 binds 1.2 equivalents of Zn(II) and that YkgM binds no Zn(II). Spectroscopic and mutagenesis studies on L31 suggest that the Zn(II) binding motif of L31 contains one cysteine at position 16 in the amino acid sequence, one histidine residue, and two nitrogen or oxygen ligands. Nonetheless, an examination of

L31 when bound to the ribosome cast doubts that L31 serves as a C+ member. By using previous crystal structures, an alternative C+/C− paralogous pair is identified.

2. Experimental procedures

2.1. Cultures for qRT-PCR studies and DNA microarrays

Nine 1-liter flasks of minimal medium [22] were inoculated with *E. coli* BW25113 (obtained National BioResource Project, National Institute of Genomics, Japan) cells from an overnight preculture and grown to an OD₆₀₀ of 0.55–0.60, at which time 3 flasks were made 10 μM in TPEN and 3 flasks were left as controls. Samples (5 mL) were extracted from each flask at 0, 0.5, 1, 5, 30, 60, 120, and 240 minutes after adding TPEN, immediately frozen in liquid nitrogen, and stored at −80 °C. For cDNA array studies, *E. coli* BW25113 cells were grown at 37 °C with continuous agitation at 200 RPM in a defined medium described previously [17]. This medium contained 10 mM glycerol as the sole carbon source. The glycerol was added to a solution containing 40 mM MES (morpholineethanesulfonic acid), 19 mM NH₄Cl, 13 mM KCl, 5.0 mM K₂SO₄, 68 μM CaCl₂, and trace elements (19 μM FeCl₃·6H₂O, 600 nM ZnO, 400 nM CuCl₂·2H₂O, 344 nM Co(NO₃)₂·6H₂O, 16 μM H₃BO₃ in distilled water). After the pH was adjusted to 7.4 and the solution was autoclaved, the solution was made 1 mM in MgCl₂ and 7.6 mM in β-glycerophosphate (as a phosphate source). Cultures were grown to mid log phase (OD₆₀₀ = 0.5), and cells were treated with 10 μM TPEN. Cells were shaken at 37 °C for 30 min and harvested for total RNA extraction. OD₆₀₀ values for TPEN-treated and control cultures were 0.56 and 0.58, respectively.

2.2. qRT-PCR studies

A RNeasy mini kit (Qiagen) was used to isolate RNA following the manufacturer's instructions. Further purification was accomplished using the RNeasy MinElute Cleanup Kit (Qiagen). The samples were then subjected to genomic DNA digestion using RNase-free DNase (Ambion). Purified and DNA-free RNA was then quantitated using a NanoDrop UV-VIS spectrophotometer. All primers except for 16S RNA were designed using PrimerQuest software from the Integrated DNA Technologies (IDT) website (<http://www.idtdna.com>). Primers obtained from IDT were: 16S RNA 5': 5'ATTGACGTTACCCGAGAAGA 3'; 16S RNA 3': 5'GGGATTTACATCCGACTTGA 3'; yodA 5': 5'GGCTGTTGCTTTAGGTGTCT 3'; YodA 3': 5'TTTGCCGTGTGAGTGATG 3'; ykgM 5': 5'GTCTCTTCTAAATCGCACCC 3'; ykgM 3': 5'TCCTTCTGATGCCACTGTTC 3'; znuA 5': 5'ATTACCAGGAGCGAAGCA 3'; znuA3': 5'TCATCAGTAGCGGTTTCACA 3'; zntA5': 5'CACAGGTTGCTGAACCTCGCC 3'; zntA3': 5'TCAATGCCAGACCCGACCA 3'; zupT5': 5'GCTGAAGGAATGTGCCT 3'; zupT3': 5'ATAGCCAAGCAGACCGAA 3'; zitB5': 5'CGCACTCACTCACACAC 3'; zitB3': 5'CATAACAAGCGACGAGCA 3'. One-step, real-time PCR was accomplished using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad). Reactions included 12.5 μL SYBR Green reaction mix, 1 μL reverse transcriptase, 100 ng RNA, 50–150 nM primers, and H₂O up to a 25 μL total reaction volume. Reactions were run using a RotorGene 3000 thermocycler. Standard curves were run for each gene, using 0.5–200 ng RNA, and each reaction was conducted in triplicate. A standard curve was then prepared by plotting the cycle threshold (Ct) vs. log ng RNA. All experiments were conducted with (−)RT controls, and values for RNA amounts were then determined using the standard curve. Fold changes were determined by comparing the TPEN-stressed samples to control samples. Standard deviations for each data point were obtained from the actual RNA amounts determined by the standard curve method for each sample.

2.3. cDNA microarrays

Total RNA was extracted using the Qiagen RNeasy Mini kit according to the manufacturer's protocol. The isolated RNA was then subjected to RT-PCR using the *znuA* gene to ensure that the *znuA* gene had been up-regulated as in previous experiments. This control was important to ensure that the growth medium did not affect expression levels of the genes. cDNA synthesis, fragmentation and labeling, and washing and scanning of *E. coli* GeneChip arrays were performed according to the instructions of the manufacturer (Affymetrix Technical Manual, Affymetrix, Inc., USA). Biotin-labeled fragmented cDNA (2.0 µg) was hybridized to *E. coli* antisense genome arrays (Affymetrix) at 45 °C for 16 h as recommended in the GeneChip technical manual (Affymetrix). The data were collected by using Affymetrix GeneChip Scanner 3300. Array hybridizations were carried out for three RNA samples prepared from three independent cultures. Intra-chip normalizations were performed, and manipulation and filtering of the data were conducted as previously described [23]. Background-subtracted data sets were used to calculate up-regulated and down-regulated genes based on fold changes of greater than 2.

2.4. Cloning, over-expression, and purification of YodA, YkgM, and L31

Two different primer sets were designed to amplify from *E. coli* genomic DNA the coding region of YodA and to introduce *NdeI* and *HindIII* restriction sites flanking the gene. The first primer set replaced the native leucine-encoding start codon with a methionine-encoding codon: MBPYodAstart: 5'-CATATGGCGATTTCGTCTTA-3' and MBPYodAterm: 5'-AAGCTTCAATGAGACATCATTTCTC-3'. The second primer set left the native leucine-encoding start codon YodAFWDMBP: 5'-CATATCTTGGCGATTTCGTCTTA-3'. The *ykgM* gene was amplified from genomic *E. coli* DNA from the BL21(DE3) strain using the forward primer 5'-AAA AAA ACA TAT GAA AAT GTA CGT GGT ATC -3' and reverse primer 5'-AAA AAG CTT ATG CCC TGC CCT CGC AG -3', respectively, and these primers introduced *NdeI* and *HindIII* restriction sites flanking the *ykgM* gene. The *L31* gene was cloned from genomic *E. coli* DNA from the BL21(DE3) strain using the following forward and reverse primers, 5'- AAA AAA ACA TAT GAA AAA AGA TAT TCA CCC G -3' and 5'- AAA AAG CTT TTA TTT GCT GCC CGG GAT GT -3', respectively, and these primers introduced *HindIII* and *NdeI* restriction sites flanking the *L31* gene. The resulting PCR products were digested with *NdeI* and *HindIII* and ligated into digested pIAD14 [24] to generate pIAD14-YodA, pIAD14-L31 and pIAD14-YkgM, respectively. The genes were verified with DNA sequencing.

The over-expression plasmids for YodA, YkgM, and L31 were transformed into BL21(DE3) *E. coli* cells. In the case of YodA, the over-expression plasmid was also transformed into BL21-CodonPlus^R (DE3)-RIPL and RosettaTM2(DE3) cells. These cells were grown in LB medium supplemented with 25 µg/mL of kanamycin at 37 °C to an OD_{600nm} of 0.8–1.0, and protein production was induced by making the cultures 0.20 mM in isopropyl-β-*D*-thiogalactopyranoside (IPTG). The cultures were shaken for three hours at 37 °C. The cells were harvested by centrifugation for ten minutes at 6831 × g. The cell pellets were resuspended with 20 mL of ice-cold, 20 mM Tris-Cl, pH 7.6, containing 200 mM NaCl. The cells were ruptured by three passes through a French pressure cell at 16,000 psi. The cell extracts were centrifuged at 23,419 × g for twenty five minutes

The supernatants were loaded at 4 °C onto Amylose columns (16 × 20 mm), which were preequilibrated with 20 mM Tris-Cl, pH 7.6, containing 200 mM NaCl. Bound proteins were eluted from the columns using 20 mM Tris-Cl, pH 7.6, containing 200 mM NaCl and 10 mM maltose. The fractions containing protein of the correct molecular weight were collected and concentrated using an Amicon ultrafiltration unit equipped with a YM-10 nitrocellulose membrane. The concentrated proteins were flash-frozen in liquid nitrogen and

stored at -80°C until further use. The purified proteins were quantitated using their absorbances at 280 nm. The molar extinction coefficients of $99,710\text{ M}^{-1}\text{cm}^{-1}$ for MBP-YodA, $71,120\text{ M}^{-1}\text{cm}^{-1}$ for MBP-YkgM, and $64,720\text{ M}^{-1}\text{cm}^{-1}$ for MBP-L31 were calculated from the amino acid compositions using the protein calculator (<http://www.scripps.edu/~edputnam/protcalc.html>).

2.5. Metal analyses

Purified proteins were diluted to $20\text{ }\mu\text{M}$ with deionized water and analyzed for metal, as described below (called as-isolated samples). To create Zn(II)-loaded samples, 1 mL of $300\text{ }\mu\text{M}$ purified protein was incubated with ten equivalents of Zn(II) for thirty minutes at 4°C , and the resulting mixture was dialyzed against $2 \times 2\text{ L}$ of 20 mM Tris-Cl, pH 7.6, for eight hours each. The Zn(II)-loaded samples were diluted to $20\text{ }\mu\text{M}$ with deionized water. The metal content of the samples was determined using a Thermo Jarrell Ash ICP-AES spectrometer, using an emission wavelength of 213.856 nm . Metal analyses were conducted in triplicate. Calibration curves were generated with the use of at least four standards, with correlation coefficients of greater than 0.999, and these calibration curves were used to determine the Fe(II), Ni(II), Mn(II), and Zn(II) content in the samples.

2.8. Site-directed mutagenesis studies

The site-directed mutagenesis of L31 was conducted using the Stratagene QuikChange site-directed mutagenesis kit, according to manufacturer's instructions. The cysteine residue present at amino acid position 16 was changed to a serine residue to produce the C16S pIAD14-L31 plasmid using the following forward and reverse mutant primers, respectively: 5'- AAA TAC GAA GAA ATT ACT GCT AGC TCG TCT TGC GGT AAC GTA ATG -'3 and 5'- CAT TAC GTT ACC GCA AGA CGA GCT AGC AGT AAT TTC TTC GTA TTT -'3. The cysteine residue present at amino acid position 18 was changed to a serine residue to produce the C18S pIAD14-L31 plasmid using the following forward and reverse mutant primers, respectively: 5'- GAA ATT ACT GCT AGC TGC TCT TCG GGT AAC GTA ATG AAA ATC CGC -'3 and 5'- GCG GAT TTT CAT TAC GTT ACC CGA AGA GCA GCT AGC AGT AAT TTC -'3. The cysteine residues present at amino acid positions 37 and 40 were changed to serine residues to produce the C37/40S pIAD14-L31 plasmid using the following forward and reverse mutant primers, respectively: 5'- CTG AAC CTC GAC GTG TCG AGC AAG TCG CAC CCG TTC TTC ACT GGC -'3 and 5'- GCC AGT GAA GAA CGG GTG CGA CTT GCT CGA CAC GTC GAG GTT CAG -'3. The C16S, C18S, and C37/40S pIAD14-L31 plasmids were transformed into *E. coli* BL21(DE3) cells as described above. The mutants were verified by DNA sequencing.

2.9. EXAFS studies

Samples of Zn(II)-supplemented L31 ($\sim 800\text{--}900\text{ }\mu\text{M}$, including 20% (v/v) glycerol added as a glassing agent) were loaded in Lucite cuvettes with $6\text{ }\mu\text{m}$ polypropylene windows and frozen rapidly in liquid nitrogen. X-ray absorption spectra were measured at the National Synchrotron Light Source (NSLS), beamline X3B, with a Si(111) double crystal monochromator; harmonic rejection was accomplished using a Ni focusing mirror. Fluorescence excitation spectra for all samples were measured with a 13-element solid-state Ge detector array. Samples were held at $\sim 15\text{ K}$ in a Displex cryostat during XAS measurements. X-ray energies were calibrated by reference to the absorption spectrum of the cobalt metal foil, measured concurrently with the protein spectra. All of the data shown represent the average of ~ 12 total scans, from two independently prepared samples each. Data collection and reduction were performed according to published procedures [25] with E_0 set to 9675 eV . The Fourier-filtered EXAFS were fit to Equation 1 using the nonlinear least-squares engine of IFEFFIT that is distributed with SixPack. Sixpack is available free of charge from its author, Sam Webb, at

<http://www-ssrl.slac.stanford.edu/~swebb/sixpack.htm>. IFEFFIT is open source software available from <http://cars9.uchicago.edu/ifeffit>.

$$\chi(k) = \sum \frac{N_{as} A_s(k) S_c}{k R_{as}^2} \exp(-2k^2 \sigma_{as}^2) \exp(-2R_{as}/\lambda) \sin[2k R_{as} + \varphi_{as}(k)] \quad (1)$$

In Eq. 1, N_{as} is the number of scatterers within a given radius ($R_{as} \pm \sigma_{as}$), $A_s(k)$ is the backscattering amplitude of the absorber-scatterer (as) pair, S_c is a scale factor, $\varphi_{as}(k)$ is the phase shift experienced by the photoelectron, λ is the photoelectron mean free-path, and the sum is taken over all shells of scattering atoms included in the fit. Theoretical amplitude and phase functions, $A_s(k) \exp(-2R_{as}/\lambda)$ and $\varphi_{as}(k)$, were calculated using FEFF v. 8.00 [26]. The scale factor (S_c) and ΔE_0 for Zn-N (0.78, -16 eV) and Zn-S (0.85, -16 eV) scattering were determined previously and held fixed throughout this analysis [25]. [25, 27] [25, 27] Fits to the current data were obtained for all reasonable integer or half-integer coordination numbers, refining only R_{as} and σ_{as}^2 for a given shell. Multiple scattering contributions from histidine ligands were approximated according to published procedures, fixing the number of imidazole ligands per metal ion at half-integral values while varying R_{as} and σ_{as}^2 for each of the four combined ms pathways (see Table 4) [25].

3. Results

3.1. Determining optimal stress time with quantitative real-time PCR (qRT-PCR)

In an effort to determine the optimal time for transcription of Zn(II)-responsive genes to begin, *E. coli* BW21135 cells were stressed with Zn(II) deficiency by using 10 μ M TPEN and harvested at various time points following the stress. The time-dependent transcription of most genes (*znuA*, *zntA*, *zitB*, and *zupT*), previously implicated in Zn(II) trafficking [1, 7, 9, 11, 28–30] in *E. coli* or regulated by Zur (*znuABC*, *ykgM* and *yodA*) [17, 31], were evaluated by using qRT-PCR. The response of zur-regulated genes (*znuA*, *ykgM*, *yodA*) to TPEN was largest at 30 minutes, showing at least a 300-fold increase for all genes (Figure 1). Previous cDNA array studies were conducted at 5 minutes and 5 hours [16, 17, 31]. Figure 1 shows that the transcription levels for the Zur-regulated genes at these times are approximately equal but are still over 100-fold less than at 30 minutes.

In contrast, the transcription levels of *zupT*, *zntA*, *zitB*, and control 16S RNA were not greatly affected by the presence of TPEN (4-fold) (data not shown), suggesting that low levels of intracellular Zn(II) do not greatly affect the transcription of these genes. The absence of a strong response of *zntA* to TPEN is consistent with previous studies on ZntR [16], which binds Zn(II) and activates production of the Zn(II) exporter ZntA. In low Zn(II) conditions, ZntR does not bind to its promoter site or to Zn(II) and is degraded [32].

3.2. cDNA microarrays

The purpose for the aforementioned real-time PCR experiments was to determine the best time to harvest cells for cDNA microarray analysis of the Zn(II) starved condition. We prepared total RNA samples from *E. coli* that were stressed for 30 minutes with TPEN and completed the subsequent microarray studies. After filtering criteria were implemented (see Experimental procedures), 114 genes were found to be up-regulated, and 146 genes were down-regulated. The top 20 up-regulated genes are shown in Table 1. In our previous microarray studies, we identified 55 up-regulated transcripts [17], and 56% of these genes were also up-regulated in this study reported here. Most of the genes found in both studies are Zur- or Fur-regulated. All five Zur-regulated genes *yodA*, *ykgM*, *znuA*, *znuB*, and *znuC* were significantly ($P < 0.05$) up-regulated in the cells stressed with TPEN for 30 minutes. Of

the remaining up-regulated genes, 22% of them belong in the inorganic ion transport and metabolism COG (cluster of orthologous group) [33], 9% have unknown function, 17% do not belong to an identified COG, and the remaining genes are spread among the other COG's (see Supplementary Materials).

Our previous microarray study only identified 46 down-regulated genes [17], while the current study revealed a 3-fold increase in the number of down-regulated genes. The top 20 down-regulated genes are shown in Table 2. Only 32% of the genes in the former study were identified in the current data, and most of the genes found in both studies are involved in flagella biosynthesis. ZntA, the high specificity exporter of Zn(II) [32], was down-regulated (5.6-fold), and this value is similar to that found in the qRT-PCR studies (*ca.* 4-fold drop). Of the remaining genes that were down-regulated in *E. coli* cells stressed for 30 minutes with TPEN, 33% are members of the energy production and conversion COG, 16% belong to the cell motility COG flagella biosynthesis), 14% belong to the amino acid transport and metabolism COG, 5% are not a member of any identified COG, and the remaining genes are spread among the other COG's [33] (see Supplementary Materials).

3.3. Over-expression, purification, and characterization of Zn(II)-responsive proteins

The three genes that exhibited the largest up-regulation in Zn(II) starvation conditions were *ykgM*, *yodA*, and *znuA* (Table 1). We previously reported structural information on ZnuA [34], which is the periplasmic component of the ABC transporter ZnuABC that has been reported to be a high-specificity Zn(II) importer in *E. coli*. Our data and others show that *E. coli* ZnuA tightly binds 2 equivalents of Zn(II), and one of the sites has a single amino acid ligand identified [34, 35]. The binding of Zn(II) causes a conformational change in the protein, which may be involved in Zn(II) transfer from ZnuA to the membrane-spanning ZnuB.

YodA is also a periplasmic protein and has been implicated in cadmium detoxification [36, 37]. We attempted to over-express and purify YodA from pET26b using BL21(DE3) *E. coli* cells, but the protein was not expressed at high levels, presumably due to the presence of 8 rare codons in the ORF of *yodA* [36]. The use of BL21-CodonPlus®(DE3)-RIPL or Rosetta™2(DE3) *E. coli* cells did not result in better over-expression of the protein. However, the over-expression and purification of YodA was greatly improved if YodA was over-expressed as a maltose binding protein (MBP) fusion protein (MBP-YodA), and five milligrams of MBP-YodA could be purified per 1 L of LB growth medium. As isolated, MBP-YodA was found to bind 0.4 equivalents of Zn(II) per protein; yet, if the purified fusion protein was incubated with 10 molar equivalents of Zn(II) and dialyzed extensively versus 30 mM Tris-HCl, pH 7.6, the resulting protein bound 4.4 ± 0.3 equivalents of Zn(II). This result is in agreement with the crystal structure of YodA, which clearly showed four Zn(II) binding sites in the protein [36]. This result does not support a previous prediction that YodA binds only 2 of the Zn(II) ions tightly [15]. The physiological role of YodA is not clear; however, it has been hypothesized that YodA is part of an ABC transport system [36]. The B and C components of this putative ABC transporter have not yet been identified.

Our initial efforts to over-express, purify, and characterize YkgM involved cloning the gene for *ykgM* into pET26b. However, soluble YkgM could not be produced from this system. Therefore, YkgM was over-expressed as part of a MBP-fusion protein, and the resulting fusion protein was purified using a single affinity (amylose) column. This strategy yielded 70 mg of >90% pure protein per liter of growth medium. Thrombin cleavage of the fusion protein resulted in the precipitation of YkgM; therefore, we were forced to analyze the MBP-YkgM fusion protein for metal content. Metal analyses demonstrated that as-isolated MBP-YkgM binds less than 0.01 equivalents of Zn(II). The purified fusion protein was incubated with 10 molar equivalents of Zn(II), and the resulting mixture was dialyzed

extensively versus metal-free, 25 mM Tris-Cl, pH 7.6, containing 200 mM NaCl. The “metal-loaded” MBP-YkgM bound less than 0.01 equivalents of Zn(II).

3.4. Over-expression, and purification of ribosomal protein L31

As mentioned in the Introduction, YkgM is hypothesized to be a non-Zn(II) binding paralog of ribosomal protein L31. Using results on *B. subtilis* as an analogy, the C[−] YkgM is produced in Zn(II) starvation conditions and replaces the C⁺ L31 on the ribosome, thereby releasing the Zn(II) bound to L31 [20, 38, 39]. To evaluate whether L31 is in fact a Zn(II)-binding protein, the *L31* gene was ligated into pIAD14 [24], which resulted in the production of a MBP-L31 fusion protein. The greatest level of over-expressed MBP-L31 was obtained by allowing the bacterial cultures to grow to an O.D._{600nm} of 0.8–1.0 before induction and by inducing protein production for 3 hours at 37 °C with 0.2 mM IPTG. Recombinant MBP-L31 was obtained by using a single affinity (amylose) column, and 70 mg/L of >95% pure protein was obtained. Efforts to remove the MBP tag resulted in irreversible protein precipitation.

Metal analyses demonstrated that MBP-L31, directly after purification, binds 0.3 ± 0.1 equivalents of Zn(II) (Table 3). No other metals were detected in significant amounts. To further probe for Zn(II) binding, MBP-L31 was incubated with up to 10 equivalents of Zn(II), and the resulting fusion protein was dialyzed versus 2 changes of 2 L of 25 mM Tris-Cl, pH 7.6, containing 200 mM NaCl. The resulting, metal-loaded MBP-L31 was shown to bind 1.2 ± 0.1 equivalents of Zn(II) (Table 3).

3.5. Site-directed mutants of MBP-L31

Previous studies on *B. subtilis* have suggested a Zn(II) starvation system in which RpmE/L31 (contains 4 Cys and is called the C⁺ protein) is replaced on the ribosome by its paralog YtiA (does not contain Zn(II)-binding Cys and is called the C[−] protein), and the Zn(II)-bound to L31 is available to the cell in Zn(II) starvation conditions. Metal analyses on L31 and YkgM suggest that these proteins might play a similar role in *E. coli*. There are four cysteines in *E. coli* L31 at positions 16, 18, 37, and 40. To probe for metal binding cysteines in *E. coli* L31, single amino acid mutations were introduced at cysteine 16 and 18 to generate the C16S and C18S single mutants of MBP-L31. A double amino acid mutation was introduced at cysteine 37 and 40 to generate the C37/40S double mutant of MBP-L31. All the single and double mutants were over-expressed, purified, and shown to be isolated as soluble fusion proteins at yields similar to those of the wild-type fusion proteins. Metal analyses on the MBP-L31 mutants were conducted to probe whether any of these cysteines participate in metal binding. The as-isolated recombinant C16S mutant contained no Zn(II) (Table 3). The C16S mutant was incubated with 10 equivalents of Zn(II), and the resulting protein solution was exhaustively dialyzed versus 2 changes of 2 liters of 25 mM Tris-Cl, pH 8.5, containing 200 mM NaCl. The “metal-loaded” C16S mutant contained 0.4 ± 0.1 equivalents of Zn(II) (Table 3). The as-isolated C18S mutant contained no Zn(II) (Table 3), and the “metal-loaded” C18S mutant bound 1.1 ± 0.1 equivalents of Zn(II) (Table 3). The as-isolated C37/40S double mutant contained 0.4 ± 0.1 equivalents of Zn(II), and the “metal-loaded” C37/40S double mutant bound 1.3 ± 0.1 equivalents of Zn(II) (Table 3).

3.6. EXAFS of MBP-L31

In an effort to characterize the metal binding site of MBP-L31, the Zn(II)-supplemented analog of MBP-L31 was examined using X-ray absorption spectroscopy (Figure 2, top). The data (solid, black line) is best modeled (open symbols) with four donors in the primary coordination sphere of the Zn(II), only one of which is sulfur (Figure 2, top, and Table 4; detailed fits are presented in Figure S1 and Table S1). Multiple scattering fits that model the

outer shell scattering of coordinated imidazole suggest that only one ligand is a histidine side chain, leaving the other two as either backbone amides or solvent.

A nearly identical picture arises when examining the EXAFS of the C37/40S double-mutant. A direct comparison of the EXAFS FTs (Figure 2, center) shows that the main peak is significantly higher in magnitude, which could reflect a slightly higher coordination number for the mutant. All of the outer shell features are nearly identical, although again the two outermost peaks are slightly higher in magnitude. Despite these small cosmetic differences, the data for the mutant are best fitted with the same model as the recombinant protein (Figure 2, bottom, and Table 4; detailed fits are presented in Table S1 and Figure S2), including a single sulfur donor in the primary coordination sphere and one coordinated imidazole. What is clear from the EXAFS is that the mutations failed to significantly alter the metal binding site.

4. Discussion

In an effort to understand intracellular Zn(II) homeostasis in *E. coli*, we and others performed proteomic and genomic studies to probe for Zn(II)-responsive proteins [13–18], reasoning that some of the proteins that were up-regulated during Zn(II) starvation would be involved with Zn(II) import, trafficking, and possibly export. While there have been great advances in cDNA array technology that make it much easier and more economical to use, the duration of the stress and type of microarray chip used remains common problems when comparing data from different cDNA microarray experiments. For any given experiment, the timing of the stress is often chosen arbitrarily. For cDNA microarrays to be performed at a vast number of time points as well as conditions introduces an enormous amount of time and expense into the equation. In previous DNA array experiments, we reported 55 up-regulated and 46 down-regulated genes in response to 5 hr of TPEN stress [17]. It is possible that some transcripts, such as those encoding for other Zn(II) transport systems such as ZitB, ZupT, and possibly ZraP, may not have exhibited differential expression because of the long stress time. One major objective of this present work was to determine an optimal time in which to perform cDNA microarray experiments when examining the effect of Zn(II) deficiency and excess on transcription in *E. coli*.

qPCR studies demonstrated that the expression of Zur-regulated genes up-regulated by low Zn(II) conditions is maximal at 30 minutes (Figure 1). Using 30 minutes of TPEN stress, we observed 114 up- and 146 down-regulated, Zn(II)-responsive genes in DNA array experiments. Many of them were observed previously [17], such as *znuA*, *ykgM*, *yodA*, and many Fur-regulated genes were up-regulated. In this work, we report two additional genes that are up-regulated by Zn(II)-starvation that are in the same gene clusters as *yodA* and *znuA* and could possibly be regulated by Zur. The first of these is *yodB*, an integral membrane protein that is a predicted cytochrome, which is located directly downstream of *yodA* in the *E. coli* chromosome. The other Zur-regulated candidate, *yebA*, is located directly downstream of *znuA*, a known Zur-regulated protein. Neither of these genes have been studied in enough detail to know if the proteins that they encode could possibly be involved in Zn(II) homeostasis and thus will be a target of future research. It is noteworthy that regulation of the other known and putative Zn(II) transporters is not observed here, other than for that of the exporter *zntA* gene (down 5.7-fold), which is transcriptionally-regulated by ZntR. The most likely reason for this result is that ZnuABC is the most important importer of Zn(II) at low Zn(II) conditions. It is also likely that intracellular Zn(II) levels are controlled primarily by import, rather than export, at least under Zn(II)-limiting conditions.

The cDNA array study did reveal, in agreement with our previous study [17], that the most up-regulated genes were *ykgM*, *yodA*, and *znuA*. We previously reported structural and metal binding studies on ZnuA, and ZnuA is known to be the periplasmic component of the high affinity Zn(II) importer ZnuABC [34]. The transcription of *znuABC* is under control of Zur, and under low Zn(II) conditions, high levels of ZnuABC are produced, and Zn(II) is imported from the periplasm of *E. coli* into the cytoplasm [31]. Previous studies showed that ZnuA can “pull” Zn(II) from other Zn(II)-containing periplasmic proteins [40]. The reason that *znuA* (and *znuB,C*) is up-regulated in our studies is clear.

The transcription of *yodA* is also under control of Zur [37], and its up-regulation during Zn(II)-deficient conditions is not surprising. Nonetheless, it is not clear exactly what role YodA plays in Zn(II) homeostasis. Previously, it was hypothesized that YodA was part of an ABC transporter system, like ZnuABC; however, the BC components of this system have not been identified [36]. Complicating this issue further is the previous suggestion that YodA is involved with Cd(II) homeostasis, possibly detoxification, and one crystal structure of YodA was obtained with 4 equivalents of Cd(II) bound [36, 37]. The crystal structure of Zn(II)-bound YodA was also reported, and this structure showed 4 Zn(II) binding sites in YodA: (1) one Zn(II) coordinated by His144 and His 155 (and two solvent molecules), (2) a second Zn(II) coordinated by His153, Glu189, and His193, (3) a third Zn(II) coordinated only by one protein ligand (His95), and (4) a fourth Zn(II) coordinated by only one protein ligand (Asp24) [36]. It was predicted that under physiological conditions that YodA only binds 2 Zn(II) ions tightly [15]. To test this hypothesis, we over-expressed and purified YodA. Due to solubility issues, we were forced to obtain YodA as a fusion protein with MBP. Metal analyses showed that YodA tightly binds 4 equivalents of Zn(II). We have attempted pulldown experiments in an effort to identify some periplasmic proteins with which YodA might interact, and several outer membrane proteins were identified [41]. However, none of the identified proteins help to explain the role of YodA in Zn(II) transport/homeostasis.

The most up-regulated gene in our studies was *ykgM*. Since the transcription of *ykgM* is under control of Zur [19], it is not surprising that the gene was up-regulated. In response to Zn(II) starvation, a Zn(II) mobilization model, called C+/C–, has been proposed in *B. subtilis*, and this model has been discussed (*vide supra*) [19]. It has been proposed that a similar system is in place in *E. coli*, and YkgM(C–)/L31(C+) were identified as a possible paralogous pair. In support of this proposal is our hypothesis that the ribosomes in *E. coli* store Zn(II) [42], and the recent confirmation that *E. coli* ribosome tightly binds up to 8 equivalents of Zn(II) (M.P. Hensley and M.W. Crowder, unpublished data). To further support the hypothesis that a C+/C– minus system is in place in *E. coli*, we over-expressed and purified YkgM and its putative paralog L31.

As with YodA, we could only obtain soluble YkgM and L31 as fusion proteins with MBP. Metal analyses demonstrate that YkgM does not bind Zn(II) and that L31 binds one equivalent of Zn(II), supporting the hypothesis that these proteins are paralogs. MBP-L31 was shown to bind 1 equivalent of Zn(II). Previously from the studies on RpmE/YtiA in *B. subtilis*, it was hypothesized that RpmE binds Zn(II) through 2–4 cysteine residues; these same residues are not found in the paralog YtiA and hence the name, C– protein [19, 20]. To probe whether Zn(II) binds any of the Cys residues in L31, EXAFS studies on the Zn(II)-supplemented analog of L31 were conducted. The best fit to the data suggests that Zn(II) binds only 1 Cys, 1 His, and 2 other low-Z (N/O) ligands. In addition, the site-directed mutagenesis data suggests that only Cys16 takes part in binding.

An examination of the crystal structure of L31 bound to the *E. coli* 50S ribosome shows that L31 binds over 100 Å from the exit tunnel (measuring the distance between Leu51 in L24

and Cys40 in L31) (Figure 3(A)). Focusing on the structure of bound L31 (Figure 3(B)), the sulfur atoms in Cys37 and Cys40 are 4.4 Å apart; however, the atoms are not facing each other perfectly for metal binding. Cys37 and Cys40 are 45 Å away from Cys16 and Cys18, which have their sulfur atoms almost 11 Å apart. From this structure, it is clear to see that it is impossible for all four cysteines to coordinate Zn(II) in L31 when it is bound to the ribosome (Figure 3 (C and D)). Given this structure, it would appear that Zn(II) would more likely to bind in between Cys37 and Cys40; however, there is no electron density in between these cysteines in any of the *E. coli* ribosome crystal structures that were examined, possibly because of the inclusion of EDTA and high levels of Mg(II) in buffers used when purifying *E. coli* ribosomes. While in solution, it appears that L31 binds to Cys16 and possibly His30 (the closest His to Cys16 – 23 Å away), which would cause a major structural change in the protein when not bound to the ribosome. It is not clear if Zn(II) binds to the same site when L31 is bound to the ribosome.

When examining many crystal structures of the *E. coli* ribosome for potential Zn(II) binding sites, we discovered L36, which is a small protein that binds to the large subunit. Previous NMR solution studies on L36 from *T. thermophilus*, showed that Zn(II) is bound by a common Zn finger motif, consisting of Cys27, His32, Cys11, and Cys14 (Figure 5) [43]. In a recent structure of the *E. coli* ribosome bound to an antibiotic, L36 was found to bind Zn(II) even when the ribosomes were purified using high levels of EDTA [44]. L36 is 158 Å away from the exit tunnel of the large subunit, and Zn(II) is coordinated by Cys11, Cys14, Cys27, and His33 (Figure 4). Interestingly, the Zn(II) binding ligands in both structures do not point directly at the Zn(II), and the metal-ligand distances are 2.26 – 2.47 Å (see Figure 4).

Therefore, L36 appears to be a better C+ candidate in *E. coli* (and *Thermus*). To be part of a C+/C– system however, there should be a C– paralog present in *E. coli*. A potential candidate is YkgO, which is transcriptionally-regulated by Zur and is part of the *ykgM/ykgO* operon [45]. We did not observe the up-regulation of the *ykgO* gene because this gene is not included in the Affymetrix gene chips used in these studies. Future studies will involve testing whether L36 and YkgO are Zn(II)-responsive paralogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the National Institutes of Health (GM079411 and GM093987), Miami University's Committee on Faculty Research, and Miami University's Volwiler professorship (to M.W.C.).

Abbreviations

COG	clusters of orthologous groups
RT-PCR	real-time polymerase chain reaction
TPEN	N,N,N',N'- <i>tetrakis</i> (2-pyridylmethyl)ethylenediamine

References Cited

1. Hantke K. Curr. Opin. Microbiol. 2005; 8:196–202. [PubMed: 15802252]
2. Vallee BL, Auld DS. Proc. Natl. Acad. Sci. 1990; 87:220–224. [PubMed: 2104979]
3. Vallee BL, Auld DS. Biochemistry. 1990; 29:5647–5659. [PubMed: 2200508]

4. Vallee, BL.; Auld, DS.; Joiles, P.; Jornvall, H., editors. Interface between Chemistry and Biochemistry. Basel, Switzerland: Birkhauser Verlag; 1995. p. 259-277.
5. Hantke K. Curr. Opin. Microbiol. 2001; 4:172-177. [PubMed: 11282473]
6. Lu M, Fu D. Science. 2007; 317:1746-1748. [PubMed: 17717154]
7. Hantke K. Biometals. 2001; 14:239-249. [PubMed: 11831459]
8. Outten CE, O'Halloran TV. Science. 2001; 292:2488-2492. [PubMed: 11397910]
9. Grass G, Fan B, Rosen BP, Franke S, Nies DH, Rensing C. J. Bacteriol. 2001; 183:4664-4667. [PubMed: 11443104]
10. Outten CE, Tobin DA, Penner-Hahn JE, O'Halloran TV. Biochemistry. 2001; 40:10417-10423. [PubMed: 11523983]
11. Grass G, Wong MD, Rosen BP, Smith RL, Rensing C. J. Bacteriol. 2002; 184:864-866. [PubMed: 11790762]
12. Jackson RJ, Binet MR, Lee LJ, Ma R, Graham AI, McLeod CW, Poole RK. FEMS Microbiol Lett. 2008; 289:219-224. [PubMed: 19054109]
13. Brocklehurst KR, Morby AP. Microbiology. 2000; 146:2277-2282. [PubMed: 10974115]
14. Lee LJ, Barrett JA, Poole RK. J. Bacteriol. 2005; 187:1124-1134. [PubMed: 15659689]
15. Graham AI, Hunt S, Stokes SL, Bramall N, Bunch J, Cox AG, McLeod CW, Poole RK. J. Biol. Chem. 2009; 284:18377-18389. [PubMed: 19377097]
16. Yamamoto K, Ishihama A. J. Bacteriol. 2005; 187:6333-6340. [PubMed: 16159766]
17. Sigdel TK, Easton JA, Crowder MW. J. Bacteriol. 2006; 188:6709-6713. [PubMed: 16952965]
18. Easton JA, Thompson P, Crowder MW. J. Biomol. Tech. 2006; 17:303-307. [PubMed: 17122063]
19. Nanamiya H, Akanuma G, Natori Y, Murayama R, Kosono S, Kudo T, Kobayashi K, Ogawawara N, Park S-M, Ochi K, Kawamura F. Molec. Microbiol. 2004; 52:273-283. [PubMed: 15049826]
20. Natori Y, Nanamiya H, Akanuma G, Kosono S, Kudo T, Ochi K, Kawamura F. Mol. Microbiol. 2007; 63:294-307. [PubMed: 17163968]
21. Panina EM, Mironov AA, Gelfand MS. Proc. Natl. Acad. Sci. 2003; 100:9912-9917. [PubMed: 12904577]
22. Rajagopalan PTR, Grimme S, Pei D. Biochemistry. 2000; 39:779-790. [PubMed: 10651644]
23. Gunasekera TS, Csonka LN, Paliy O. J. Bacteriol. 2008; 190:3712-3720. [PubMed: 18359805]
24. McCafferty DG, Lessard IAD, Walsh CT. Biochemistry. 1997; 36:10498-10505. [PubMed: 9265630]
25. Thomas PW, Stone EM, Costello A, Tierney DL, Fast W. Biochemistry. 2005; 44:7559-7569. [PubMed: 15895999]
26. Ankudinov AL, Ravel B, Rehr JJ, Conradson SD. Phys. Rev. B. 1998; 58:7565-7576.
27. Periyannan G, Costello AL, Tierney DL, Yang KW, Bennett B, Crowder MW. Biochemistry. 2006; 45:1313-1320. [PubMed: 16430228]
28. Beard SJ, Hashim R, Wu G, Binet MRB, Hughes MN, Poole RK. FEMS Microbiol. Lett. 2000; 184:231-235. [PubMed: 10713426]
29. Blencowe DK, Morby AP. FEMS Microbiol. Lett. 2003; 27:291-311.
30. Chimienti F, Aouffen M, Favier A, Seve M. Curr. Drug Targets. 2003; 4:323-338. [PubMed: 12699353]
31. Patzer SI, Hantke K. Mol. Microbiol. 1998; 28:1199-1210. [PubMed: 9680209]
32. Rensing C, Mitra B, Rosen BP. Proc. Natl. Acad. Sci. 1997; 94:14326-14331. [PubMed: 9405611]
33. Tatusov RL, Natale DA, Garkavtsev IV, Tatusova TA, Shankavaram UT, Rao BS, Kiryutin B, Galperin MY, Fedorova ND, Koonin EV. Nucleic Acid Res. 2001; 29:22-28. [PubMed: 11125040]
34. Yatsunyk LA, Easton JA, Kim LR, Sugarbaker SA, Bennett B, Tierney DL, Crowder MW, Rosenzweig AC. J. Biol. Inorg. Chem. 2008; 13:271-288. [PubMed: 18027003]
35. Ammendola S, Pasquali P, Pistoia C, Petrucci P, Petrarca P, Rotilio G, Battistoni A. Infect. Immun. 2007; 75:5867-5876. [PubMed: 17923515]
36. David G, Blondeau K, Schiltz M, Penel S, Lewit-Bentley A. J. Biol. Chem. 2003; 278:43728-43735. [PubMed: 12909634]

37. Puskarova A, Ferianc P, Kormanec J, Homerova D, Farewell A, Nystrom T. Microbiology. 2002; 148:3801–3811. [PubMed: 12480884]
38. Nanamiya H, Akanuma G, Natori Y, Murayama R, Kosono S, Kudo T, Kobayashi K, Ogasawara N, Park SM, Ochi K, Kawamura F. Mol Microbiol. 2004; 52:273–283. [PubMed: 15049826]
39. Nanamiya H, Kawamura F, Kosono S. J. Gen. Appl. Microbiol. 2006; 52:249–258. [PubMed: 17310068]
40. Berducci G, Mazzetti AP, Rotilio G, Battistoni A. FEBS Lett. 2004; 569:289–292. [PubMed: 15225650]
41. Sigdel, TK. A search for Zn(II) metallochaperones in *E. coli*, proteomic and genomic approaches. Ph.D. Oxford, OH: Miami University; 2005.
42. Gunasekera, TS.; Easton, JA.; Sugarbaker, SA.; Klingbeil, L.; Crowder, MW. Zn(II) homeostasis in *E. coli*. In: Long, EC.; Baldwin, MJ., editors. Bioinorganic chemistry: Cellular systems and synthetic models. Washington, D.C: American Chemical Society; 2009. p. 81–93.
43. Hard T, Rak A, Allard P, Kloos L, Garber M. J. Mol. Biol. 2000; 296:169–180. [PubMed: 10656825]
44. Dunkle JA, Xiong L, Mankin AS, Cate JH. Proc. Natl. Acad. Sci. 2010; 107:17152–17157. [PubMed: 20876128]
45. Hemm MR, Paul BJ, Miranda-Rios J, Zhang A, Soltanzad N, Storz G. J. Bacteriol. 2010; 192:46–58. [PubMed: 19734316]
46. Kehres DG, Zaharik ML, Finlay BB, Maguire ME. Mol. Microbiol. 2000; 36:1085–1100. [PubMed: 10844693]
47. Gehring AM, Mori I, Walsh CT. Biochemistry. 1998; 37:2648–2659. [PubMed: 9485415]
48. Escolar L, Perez-Martin J, de Lorenzo V. J. Bacteriol. 1998; 180:2579–2582. [PubMed: 9573216]
49. Vanderkelen L, Van Herreweghe JM, Vanoirbeek KG, Baggerman G, Myrnes B, Declerck PJ, Nilsen IW, Michiels CW, Callewaert L. Cell. Mol. Life Sci. 2011; 68:1053–1064. [PubMed: 20734102]
50. Hunt MD, Pettis GS, McIntosh M. J. Bacteriol. 1994; 176:3944–3955. [PubMed: 8021177]
51. Chen D, Wu R, Bryan TL, Dunaway-Mariano D. Biochemistry. 2009; 48:511–513. [PubMed: 19119850]
52. Liu J, Quinn N, Berchtold GA, Walsh CT. Biochemistry. 1990; 29:1417–1425. [PubMed: 2139795]
53. Strahsburg E, Baeza M, Monasterio O, Lagos R. Antimicro. Agents Chemo. 2005; 49:3083–3086.
54. Sauer M, Hantke K, Braun V. J. Bacteriol. 1987; 169:2044–2049. [PubMed: 3032906]
55. Panina EM, Mironov AA, Gelfand MS. Nucleic Acid Res. 2001; 29:5195–5206. [PubMed: 11812853]
56. Rusnak F, Sakaitani M, Drueckhammer D, Reichert J, Walsh CT. Biochemistry. 1991; 30:2916–2927. [PubMed: 1826089]
57. Liu J, Duncan K, Walsh CT. J. Bacteriol. 1989; 171:791–798. [PubMed: 2521622]
58. Braun V, Braun M. FEBS Lett. 2002; 529:78–85. [PubMed: 12354617]
59. Hopkin K, Papazian M, Steinman H. J. Biol. Chem. 1992; 267:24253–24258. [PubMed: 1447175]
60. Rodrigue A, Effantin G, Mandrand-Berthelot MA. J. Bacteriol. 2005; 187:2912–2916. [PubMed: 15805538]
61. Cheng VW, Ma E, Zhao Z, Rothery RA, Weiner JH. J. Biol. Chem. 2006; 281:27662–27668. [PubMed: 16864590]
62. Kiley PJ, Beinert H. Curr. Opin. Microbiol. 2003; 6:181–185. [PubMed: 12732309]
63. Flint DH. Arch. Biochem. Biophys. 1994; 311:509–516. [PubMed: 8203917]
64. Hussain H, Grove J, Griffiths L, Lusby S, Cole J. Mol. Microbiol. 1994; 12:153–163. [PubMed: 8057835]
65. Letoffe S, Delepelaire P, Wandersman C. Proc. Natl. Acad. Sci. 2006; 103:12891–12896. [PubMed: 16905647]
66. Ding H, Clark RJ, Lu K. J. Biol. Chem. 2004; 279:37499–37504. [PubMed: 15247288]
67. Tu AH, Turnbough CL. J. Bacteriol. 1997; 179:6665–6673. [PubMed: 9352914]

68. Mihara H, Hidese R, Yamane M, Kurihara T, Esaki N. *Biochem. Biophys. Res. Comm.* 2008; 372:407–411. [PubMed: 18482579]
69. McNicholas PM, Gunsalus RP. *J. Bacteriol.* 2002; 184:3253–3259. [PubMed: 12029041]
70. Woods SA, Schwartzbach SD, Guest JR. *Biochim. Biophys. Acta.* 1988; 954:14–26. [PubMed: 3282546]
71. Matsushita K, Ohnishi T, Kaback HR. *Biochemistry.* 1987; 26:7732–7737. [PubMed: 3122832]

Figure 1.
Time-dependent real-time PCR of Zur-regulated genes.

Figure 2.
EXAFS Fourier transforms for Zn(II)-supplemented MBP-L31 (black) and the C37S/C40S double mutant (gray).

Figure 3. Crystal structure of L31. (A) L31 bound to *E. coli* ribosome. (B) *E. coli* L31. (C) Site one of L31 containing Cys37 and Cys40. (D) Site two of L31 containing Cys16 and Cys18.

Figure 4. Structures of L36: (A) *T. thermophilus* L36 (NMR; [43]) and (B) *E. coli* L36 (crystal structure; [44])

Table 1

Top twenty genes up-regulated by TPEN

Gene	ID	Description	P value	Fold change	Regulator	Ref
<i>ykgM</i>	B0296	Paralog of ribosomal protein L31	2.0E-05	205	Zur	[19]
<i>yodA/zinT</i>	B1973	Cadmium-induced cadmium binding protein	2.0E-05	168	Zur	[36]
<i>znuA</i>	B1857	A subunit of ABC transporter	2.0E-05	42	Zur	[34]
<i>mntH</i>	B2392	Manganese NRAMP transporter	2.0E-05	33	MntR, Fur	[46]
<i>entD</i>	B0583	enterobactin synthetase, component D	9.8E-05	21	Fur	[47]
<i>entE</i>	B0594	Enterobactin synthetase, component E	2.0E-05		Fur	[47]
<i>fes</i>	B0585	Enterobactin esterase	2.0E-05	13	Fur	[48]
<i>yegK/pliG</i>	B1178	Periplasmic lysozyme inhibitor	2.0E-05	12	?	[49]
<i>fepA</i>	B0584	outer membrane receptor for ferric enterobactin and colicins B and D	2.0E-05	11	Fur	[50]
<i>yoeA</i>	B1995	Disrupted hemin or colicin receptor	2.0E-05	10	Fur	[21]
<i>yhdB/entH</i>	B0597	Proofreading thioesterase in enterobactin biosynthesis	2.0E-05	10	Fur	[51]
<i>entC</i>	B0593	isochorismate hydroxymutase 2, enterobactin biosynthesis	2.0E-05	10	Fur	[52]
<i>fiu</i>	B0805	putative outer membrane receptor for iron transport	1.2E-04	9.8	Fur	[53]
<i>fhuE</i>	B1102	outer membrane receptor for ferric iron uptake	2.0E-05	9.4	Fur	[54]
<i>ybiX</i>	B0804	unknown	2.3E-05	9.3	Fur	[55]
<i>entB</i>	B0595	2,3-dihydro-2,3-dihydroxybenzoate synthetase, isochorismatase	2.0E-05	8.6	Fur	[47]
<i>entF</i>	B0586	ATP-dependent serine activating enzyme	2.0E-05	8	Fur	[56]
<i>entA</i>	B0596	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	2.0E-05	7.9	Fur	[57]
<i>fecR</i>	B4292	regulator for fec operon, periplasmic	2.0E-05	7.5	Fur	[58]
<i>fecI</i>	B4293	probable RNA polymerase sigma factor	2.0E-05	7.3	Fur	[58]

Table 2

Genes down-regulated by TPEN:

Gene	ID	Description	P value	Fold change	Regulator	Ref
<i>sodB</i>	b1656	superoxide dismutase, iron	2.0E-05	-1.5	Fur	[59]
<i>yohM/rcnA</i>	b2106	Efflux pump for nickel and cobalt	2.1E-05	-9.9	RncR, Fur	[60]
<i>sdhD</i>	b0722	succinate dehydrogenase, hydrophobic subunit	2.0E-05	-8.6	CRP	[61]
<i>acnB</i>	b0118	aconitate hydratase B	2.0E-05	-7.2	FruR	[62]
<i>sdhA</i>	b0723	succinate dehydrogenase, flavoprotein subunit	2.0E-05	-7.1	CRP	[61]
<i>fumA</i>	b1612	fumarase A = fumarate hydratase Class I; aerobic isozyme	2.0E-05	-6.3	Fnr, ArcA	[63]
<i>sdhB</i>	b0724	succinate dehydrogenase, iron sulfur protein	2.0E-05	-6.3	CRP, ArcA	[61]
<i>nrfC</i>	b4072	formate-dependent nitrite reductase; Fe-S centers	8.0E-04	-6.2	FlhDC	[64]
<i>sdhC</i>	b0721	succinate dehydrogenase, cytochrome b556	2.0E-05	-6.1	CRP, ArcA	[61]
<i>dppB</i>	b3543	dipeptide transport system permease protein 1	2.5E-02	-5.9	Fnr or IHF	[65]
<i>dppC</i>	b3542	dipeptide transport system permease protein 2	5.4E-05	-5.7	Fnr or IHF	[65]
<i>zntA/yhhO</i>	b3469	zinc-transporting ATPase	2.0E-05	-5.7	ZntR	[32]
<i>iscS</i>	b2530	cysteine desulfurase	2.0E-05	-5.1	IscR	[66]
<i>uraA</i>	b2497	uracil transporter	2.1E-05	-4.9	Fnr	[67]
<i>yeiT</i>	b2146	Dihydrothymine dehydrogenase	2.2E-05	-4.8	CRP	[68]
<i>dppD</i>	b3541	putative ATP-binding component of dipeptide transport system	2.0E-05	-4.7	Fnr or IHF	[65]
<i>dppF</i>	b3540	putative ATP-binding component of dipeptide transport system	5.1E-05	-4.4	Fnr or IHF	[65]
<i>napG</i>	b2205	ferredoxin-type protein: electron transfer	4.7E-05	-4.3	FlhDC	[69]
<i>fumC</i>	b1611	Type II fumarase	2.0E-05	-4.3	SoxR, SoxS,	[70]
<i>nuoG</i>	b2283	NADH dehydrogenase I chain G	2.2E-05	-4.2	Fnr	[71]

Table 3

Metal analyses of MBP-L31 and MBP-YkgM and mutants

Protein	Zn(II) (equivalents)
L31 as-purified	0.3 ± 0.1
L31 metal-loaded	1.2 ± 0.1
YkgM as-purified	<0.01
YkgM metal-loaded	<0.01
L31 (C16S) as-purified	<0.01
L31 (C16S) metal-loaded	0.4 ± 0.1
L31 (C18S) as-purified	0.03 ± 0.01
L31 (C18S) metal-loaded	1.1 ± 0.1
L31 (C37S/C40S) as-purified	0.4 ± 0.1
L31 (C37S/C40S) metal-loaded	1.3 ± 0.1

Table 4Best fits to the Zn EXAFS of MBP-L31 and its C37S/C40S double mutant.^a

Sample	Zn-N/O	Zn-S	Zn-His ^b
MBP-L31	2.02 (6.8)	2.31 (5.6)	2.86 (5.9), 3.09 (1.0), 4.07 (15), 4.44 (16)
MBP-L31 (C37S/C40S)	2.01 (5.2)	2.29 (5.3)	2.88 (9.8), 3.10 (3.9), 4.12 (15), 4.42 (21)

^aDistances (Å) and disorder parameters (in parentheses, $\sigma^2 (10^{-3} \text{ Å}^2)$) shown derive from integer coordination number fits to filtered EXAFS data [$k = 1\text{--}13 \text{ Å}^{-1}$; $R = 0.1\text{--}4.0 \text{ Å}$].

^bMultiple scattering paths represent combined paths, as described previously (see Experimental procedures).