Journal of Biomolecular Techniques
17:303–307 © 2006 ABRF

COMMUNICATION

Time-dependent Translational Response of *E. coli* to Excess Zn(II)

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Zinc homeostasis is not well understood beyond methods of import and export. In order to better understand zinc homeostasis in *Escherichia coli* by identifying Zn(II)-responsive proteins, a proteomic approach was taken. Through the use of two-dimensional gel electrophoresis, we were able to show that the levels of OmpF, AspC, YcdO, Eno, and CysE increased after 30 min of Zn(II) stress, while the levels of Tig, TufA, SelA, and LeuC decreased relative to non-stressed controls. After 4 h of Zn(II) stress, the levels of three proteins (DnaK, YeaU, and Mdh) were found to be up-regulated, while the levels of seven amino acid importers (HisJ, ArgT, LivJ, DppA, OppA, RbsB, and GinH) were found to be decreased. None of these proteins had been reported to be up- or down-regulated in any previously published cDNA microarray experiments. This result raises questions about the validity of cDNA arrays when they are used to make assumptions concerning protein levels within bacterial cells. These data also suggest that time is a factor when characterizing how the *E. coli* proteome responds to Zn(II) stress.

KEY WORDS: Zn(II), 2D gels, translational response.

Zinc (Zn[II]) is an essential trace element required for life in all organisms.[1] Zn(II) has been identified as a structural and/or catalytic cofactor in all six major classes of proteins.[2,3] Recent findings by several groups have probed cellular response to zinc stress using cDNA microarrays.[4–6] These studies differed in experimental conditions such as growth media, growth conditions, and stress time. As a result, each study identified different up- and down-regulated transcripts. Selection of growth conditions, particularly stress time and medium composition, had an impact on the outcome of these studies. Yamamoto et al.[6] and Brocklehurst et al.[4] used Luria-Bertani medium, while Lee et al.[5] used glycerophosphate-containing minimal medium. Lee et al. used chemostat-regulated cultures,[5] while Brocklehurst et al. used zinc-adapted cultures.[4] It cannot be assumed that the same genes will be up- or down-regulated at various times throughout the duration of a stress. To our knowledge, no proteomic studies have been reported that probed the response of *E. coli* to Zn(II) stress, nor are there studies examining whether the translational response is dependent on incubation time. There is a recent report describing a proteomic study on the adaptive response of *E. coli* to elevated (non-stress-inducing) levels of Zn(II).[7] In the present study, we have utilized two-dimensional gel electrophoresis along with peptide identifications in an effort to probe the differences between short-term and long-term response to Zn(II) stress and to compare these proteomic results to previously reported genomic results.

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MATERIALS AND METHODS

A preculture of BL21(DE3) *E. coli* was grown overnight in glycerophosphate-containing minimal medium prepared as previously described.[5] The preculture was then split into six cultures, and all six were allowed to reach an OD_{600} of 0.55–0.59. Three cultures were labeled as control, and the other three were made 0.2 mM $ZnSO_4$. The cells were cultured at 37°C for 30 min or 4 h, and growth curves were obtained for all cultures. After growth, the cells were harvested via centrifugation at 7000 *g* for 15 min and then lysed via ultrasonic dismembration. The soluble fractions were collected via centrifugation at 30,000 *g* for 30 min. Total protein was quantitated at $\lambda = 280$ nm using a NanoDrop UV/Vis spectrophotometer. The samples were then analyzed using two-dimensional gel electrophoresis as described previously using 11-cm IPG strips (pH 4–7),[8] and all gels were run in triplicate. The second dimension

TABLE 1

Proteins with Differential Expression after Zn(II) Stress for 30 min

Protein	Gene	ID[a]	Description	Fold change in excess Zn(II)	Ref.
OmpF	ompF	B0929	Outer membrane porin F	2.1	9
AspC	*aspC*	B0928	Aspartate aminotransferase	2.5	10
Tig	*tig*	B0436	Trigger Factor	–2.1	15
YcdO	*ycdO*	B1018	Hypothetical protein	2.2	11,12
TufA	*tufA*	B3339	Elongation factor Tu	–3.0	17
Eno	*eno*	B2779	Enolase	1.6	13
CysE	*cysE*	B3607	Cysteine synthase	1.6	14
SelA	*selA*	B3591	Selenocysteine synthase	–1.6	18
LeuC	*leuC*	B0072	Isopropylmalate isomerase subunit	–1.8	19

[a]Blattner number

was accomplished using Criterion Precast SDS-PAGE gels (Tris-HCl, 8–16% resolving, 4% stacking). Gels were stained with Coomasie blue dye. Spots were quantitated using PDQuest software. Spots varying in intensity from gels of the experimental and control samples by a factor of at least 1.5 in at least two of the three gels were excised and subjected to in-gel trypsin digestion. Peptide identifications were accomplished as described previously.[7,8]

RESULTS AND DISCUSSION

The amount of Zn(II) required to sufficiently stress *E. coli* cells was determined using varying amounts of Zn(II) and measuring growth over time. In an effort not to severely inhibit growth to the point of cell death, we chose an amount (0.2 mM) of Zn(II) that showed some decrease in growth over control but did not completely abolish growth.[5] We used two-dimensional gel electrophoresis to identify proteins whose levels change as a result of high Zn(II) stress. In the case of the 30-min stress samples, nine gel spots exhibited differential intensities of greater than 1.5, and these spots were subjected to peptide identifications.[8] Five proteins were up-regulated (OmpF, AspC, YcdO, Eno, and CysE), and four proteins were down-regulated (Tig, TufA, SelA, and LeuC) in the presence of excess Zn(II) (Table 1). OmpF is an outer-membrane porin that allows sugars, ions, and amino acids of less than 600 Da into the periplasm. It also has weak preference for cationic molecules.[9] OmpF was shown to be up-regulated in *E. coli* cells that were grown in the presence of non-stress-inducing concentrations of Zn(II).[7] Aspartate aminotransferase (AspC) is an enzyme active in the synthesis of aspartate, tyrosine, and phenylalanine.[10] Very little is known about the hypothetical protein YcdO. Regulation has been described,[11] and it has been shown to be induced at low pH.[12] Enolase (Eno) is involved in glucose metabolism, and it has been shown to be a component of the degradosome.[13] Enolase was shown to be up-regulated in *E. coli* cells that were grown in the presence of non-stress-inducing conditions.[7] Serine acetyltransferase (CysE) is a subunit of the cysteine synthase complex that is responsible for the synthesis of *L*-cysteine from *L*-alanine.[14] Trigger factor (Tig) is a molecular chaperone that is involved in the folding of newly synthesized proteins in the cytoplasm.[15] Interestingly, trigger factor was shown to be down-regulated in *E. coli* cells that were grown in the presence of non-stress-inducing conditions.[7] Recently, Suno et al. reported that trigger factor from *Thermus thermophilus* binds 0.5 equivalents of Zn(II);[16] it is not known whether *E. coli* trigger factor binds Zn(II) or whether the prolyl isomerase or protein-folding activities require the presence of Zn(II). Elongation factor Tu (TufA) is involved in peptide elongation.[17] Selenocysteine synthase (SelA) is an enzyme involved in the production of selenocysteinyl-tRNA.[18] LeuC is a subunit of isopropylmalate isomerase that is involved in the isomerization of 3-carboxy-3-hydroxy-isocaproate to 2-*D*-threo-hydroxy-3-carboxy-isocaproate during leucine synthesis.[19]

When *E. coli* were stressed with excess Zn(II) for 4 h, different proteins were found to be up- and down-regulated (Table 2). Three proteins (DnaK, YeaU, and Mdh) were found to be up-regulated compared to controls. The levels of seven proteins (HisJ, ArgT, LivJ, DppA, OppA, RbsB, and GinH) were found to be down in Zn(II)-stressed cultures. DnaK is a molecular chaperone that, in conjunction with DnaJ and GrpE, is responsible for folding of nascent polypeptide chains, the rescue of misfolded peptides, and peptide translocation through membranes.[20] YeaU is a putative tartrate dehydroge-

TABLE 2

Proteins with Differential Expression after Zn(II) Stress for 4 h

Protein	Gene	ID[a]	Description	Fold change in excess Zn(II)	Ref.
DnaK	*dnaK*	B0014	Molecular chaperone	3.8	20
YeaU	*yeaU*	B1800	Predicted dehydrogenase	2.7	21
Mdh	*mdh*	B3236	Malate dehydrogenase	1.5	22
HisJ	*hisJ*	B2309	Component of histidine ABC transporter	–1.8	23
ArgT	*argT*	B2310	Component of lysine/arginine/ornithine ABC transporter	–1.9	24
LivJ	*livJ*	B3460	Component of branched chain amino acids ABC transporter	–1.9	25
DppA	*dppA*	B3544	Component of dipeptide ABC transporter	–1.6	23
OppA	*oppA*	B1243	Component of oligopeptide ABC transporter	–2.1	26
RbsB	*rbsB*	B3751	Component of ribose ABC transporter	–2.7	27
GlnH	*glnH*	B0811	Component of glutamine ABC transporter	decrease[b]	23

[a]Blattner number
[b]Protein was observed in control gels but not observed in experimental gels.

nase,[21] but no information is available about this protein at the time of this writing. Malate dehydrogenase (Mdh) is a TCA cycle enzyme responsible for the conversion of malate to oxaloacetate.[22] All of the down-regulated proteins in the 4-h-stressed samples were periplasmic transporters involved in transporting peptides or sugars from the periplasm into the cytoplasm. HisJ is a component of the periplasmic HisPMQJ ATP-dependent histidine transporter,[23] and HisJ was shown to be down-regulated in *E. coli* cells that were grown in the presence of non-stress-inducing conditions.[7] ArgT is a component of the periplasmic lysine/arginine/ornithine ABC transporter.[24] LivJ is a component of the periplasmic LivFGHMJ branched-chain amino acid ABC transporter.[25] DppA is a component of the periplasmic DppABCDF dipeptide transporter responsible for transporting peptides into the cytoplasm.[23] OppA is a component of the OppABCDF oligopeptide transporter responsible for transporting oligopeptides from the periplasm into the cytoplasm.[26] RbsB is a component of the RbsABC transporter responsible for the transport of *D*-ribose into the cytoplasm.[27] GlnH is a component of the GlnHPQ high-affinity glutamine transport system.[23]

Zinc homeostasis in *E. coli* has been studied extensively.[1] Two import and two export systems have been identified in *E. coli*. ZnuABC system is the high-affinity influx pump that is regulated at the transcriptional level by Zur, a protein similar to Fur, which represses transcription in the presence of Zn(II).[28] ZupT is a non-specific metal transporter also capable of importing Zn(II).[29] There are also two export systems that help control Zn(II) levels within *E. coli* cells. ZntA is an export protein that is transcriptionally regulated by ZntR, a MerR homolog that, upon Zn(II) binding, induces transcription of ZntA.[1,28] ZitB, a member of the cation diffusion facilitator family, is an exporter specifically induced by Zn(II).[30]

Zinc transport within an *E. coli* cell remains somewhat of a mystery once the Zn(II) ion leaves the inbound transporter. No cytosolic Zn(II) metallochaperones have been discovered. Microarrays have been used to probe for Zn(II)-responsive proteins under a variety of conditions, all using elevated Zn(II) levels. One study from our laboratory has used the metal ion chelator N,N,N',N'-*tetrakis*(2-pyridylmethyl)ethylenediamine (TPEN) to probe the transcriptional response to zinc deficiency.[31] In addition, a proteomics study that probed the effect of non-stress-inducing concentrations of Zn(II) on *E. coli* has been reported.[7] A proteomic approach to probe the effects of Zn(II) stress on the *E. coli* proteome has identified 9 proteins whose levels changed after 30 min of stress and 10 proteins whose levels changed after 4 h of stress. As suspected, none of the same proteins were observed in the two stress conditions. This result remains consistent with the idea that the cell has a set of mechanisms to deal with Zn(II) stress initially, and then other mechanisms as the duration of the stress increases. While there is clearly a pattern of down-regulated transporters occurring in the 4-h stressed cells, no such patterns appear in the 30-min stress. As all of the down-regulated transporters are ABC transporters, thus requiring ATP for function, it is possi-

ble that the cell conserves ATP for other functions crucial for cell survival. One such process could be the removal of excess Zn(II) by the ATP-requiring Zn(II) transporters. It is possible that other transporters (H^+ gradients) could be used to regulate intracellular Zn(II) levels.

None of the proteins observed in this study were identified in any of the genomic approaches in the literature.[4–6] This result is surprising because, while it would be reasonable to expect to see small gene expression changes due to the sensitivity of cDNA arrays, it is counter-intuitive to think that protein expression levels that are significant enough to be seen with the less-sensitive two-dimensional gels would not be seen with cDNA arrays. This raises the question of why the two techniques do not correlate. cDNA arrays have been used to study gene expression in prokaryotes, and it has been assumed that, since bacteria lack the ability for extensive post-translational modifications, it is reasonable to extrapolate those changes in mRNA abundance to protein abundance as well. In this study, this scenario seems not to be the case. A possible explanation could be that there is a considerable amount of regulation at the translational level as well as at the transcriptional level.

Of the known zinc transporters, ZntA, ZitB, ZnuBC, and ZupT are membrane associated and not detectable by the methods used here. Two soluble, periplasmic Zn-binding proteins, ZnuA and ZraP, were not observed in our two-dimensional gels. ZnuA has a molecular weight of 33 kDa and a pI of 5.98, and should have been detected using the method described here if expression levels had been high enough, or if levels had changed more than 1.5-fold relative to control. However, ZnuA is a high-affinity zinc-binding protein whose transcription is regulated by Zur, so expression would not have been increased in the experiments described here.[32] ZraP is a zinc-binding protein with a molecular weight of 15 kDa and a pI of 9.1, which is outside the detectable range of proteins in this study.[33] Membrane solubilization kits were not used in this study because they would not aid in the identification of a cytosolic Zn(II)-binding protein that could freely move about the cytosol to distribute Zn(II) ions to the areas or proteins that require them.

We conclude through the use of proteomic methods and an analysis of previously published genomic data that there appears to be no correlation between genomic and proteomic data for the response of *E. coli* to Zn(II) excess. This result is troubling and calls into question the validity of those genomic data when they are used to extrapolate mRNA levels to protein levels within cells. While our work did not reveal a candidate for a zinc metallochaperone, it does suggest that the cellular proteomic response to elevated Zn(II) levels varies with the time of exposure.

ACKNOWLEDGMENTS

This work was funded by the National Institutes of Health (GM079411 and GM40052 to M.W.C.) and Miami University (10X Postdoctoral fellowship to P.T.).

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