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Probing the adaptive response of *Escherichia coli* to extracellular Zn(II)

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Abstract

The adaptive response of *Escherichia coli* cells to differing intracellular and extracellular Zn(II) concentrations was evaluated by two-dimensional gel electrophoresis and peptide identifications. Twenty-one Zn(II)-responsive proteins, which were previously not known to be associated with Zn(II), were identified. Most of the proteins were related to cellular metabolism and include membrane transporters and glycolytic and TCA-associated enzymes. The expression levels of no known Zn(II) transporters were identified with these studies. The results of these studies suggest a role of Zn(II) in the expression levels of several *E. coli* proteins, and the results are discussed in light of recent genomic profiling studies on the adaptive response of *E. coli* cells to stress by Zn(II) excess.

Abbreviations: ABC – ATP-binding cassette; DEAE – diethylaminoethyl; ICP-AES – inductively coupled plasma atomic emission spectrometry; IEF – isoelectric focusing; IPG – immobilized pH gradient; MALDI-TOF MS – matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PMSF – phenylmethylsulfonylfluoride; TEM – transmission electron microscopy.

Introduction

Zinc is known to be an essential element for many organisms, and its role in biology has been recognized since the mid-twentieth century (Vallee 1988; Vallee & Falchuk 1993; Maret 2001; Blencowe & Morby 2003). The involvement of Zn(II) in all classes of enzymes has made it one of the most interesting metals in biological systems (Maret 2004). Recently, a role of Zn(II) in modulating the overall metabolic processes within the cell has become a new paradigm in this field of research (Vallee 1988; Coleman 1992; Gaither & Eide 2001; Hantke 2001; Blencowe & Morby 2003; Liuzzi & Cousins 2004; Maret 2004). There is considerable interest in the roles and homeostasis of Zn(II); however, the detailed mechanism on how this essential metal gets imported, transported, and exported according to the needs of the cell is still not clear (Gaither & Eide 2001; Hantke 2001, 2005; Liuzzi & Cousins 2004). The expression levels of all proteins involved in Zn(II) homeostasis should be regulated at some level by intra- and extracellular concentrations of Zn(II). Therefore, the identification of the Zn(II) proteome or Zn(II)-responsive proteins should aid in better understanding Zn(II) homeostasis in cells. There are two reports that described the transcriptional, adaptive response of *Escherichia coli* to excess Zn(II) (Brocklehurst & Morby 2000; Lee

et al. 2005). However, there have been no reports describing any proteomic profiling of Zn(II)-responsive proteins in *E. coli* cells.

In this study we utilized two-dimensional gel electrophoresis/peptide identifications to probe for the adaptive response of *E. coli* to differential concentrations of intra- and extracellular Zn(II) and to identify Zn(II) responsive proteins. The level of Zn(II) used was chosen as to not stress the cells, and growth curves and microscopy demonstrated no discernable differences in phenotypes between control and experimental cell cultures. These studies identified several proteins, which were previously not associated with Zn(II) and are associated with cellular stress.

Experimental section

Materials

Diethylaminoethyl (DEAE) Sepharose was purchased from Amersham Biosciences (Piscataway, NJ). D-(+)-Glucose and bovine serum albumin were procured from Sigma (St. Louis, MO). All other chemicals were obtained from Fisher Scientific (Liberty LaneHampton, NH). Immobilized pH gradient strips, carrier ampholytes, ReadyPrep 2-D Cleanup kit, Bio-Rad protein assay dye, and Criterion precast gels were purchased from Bio-Rad (Hercules, CA).

Strain and growth conditions

The BL21(DE3) strain of *E. coli* was obtained from Invitrogen (Carlsbad, CA). Two 50-ml cultures of *E. coli* BL21(DE3) were grown overnight in minimal media (Rajagopalan *et al*. 2000) containing 250 μM added Zn(II) or containing no added Zn(II) at 37°C. The minimal media contained 2.5 g/l D-(+)-glucose, 5 g/l casamino acids, 10.8 g/l K_2HPO_4, 5.5 g/l KH_2PO_4, 10 g/l NaCl, 1 g/l $(NH_4)_2SO_4$, 2 mg/l thiamine, 1 mg/l biotin, 124 mg/l $MgSO_4{\cdot}7H_2O$, 74 μg/l $CaCl_2{\cdot}2H_2O$, 20 μg/l $MnCl_2{\cdot}4H_2O$, 31 μg/l H_3BO_3, 1.2 μg/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 1.6 μg/l $CuSO_4$. These precultures were used to inoculate 8× 1-l cultures containing minimal media and minimal media with 250 μM added Zn(II). Cells were grown by shaking the culture flasks at 37°C for 3 h and were harvested by centrifugation for 15 min at 8275 × *g*. The cell pellets were resuspended in 15 ml of 50 mM Tris–HCl, pH 7.5, and lysed by two passages through a French press at 16,000 psi. The cell debris was removed by centrifugation for 15 min at 32,583 × *g*. A Bradford protein assay was used to quantify proteins. Fractionation of cell cultures with DEAE Sepharose and preparation of the 2D gel samples have been described elsewhere (Sigdel *et al*. 2004).

Imaging using transmission electron microscopy (TEM)

Escherichia coli cultures in minimal media with 250 μM Zn(II) and without added Zn(II) were grown at 37°C until reaching an optical density at 600 nm of 0.6. Bacterial cells were negative stained with 1.5% (w/v) ammonium molybdate on carbon coated, collodion-substrated 300 mesh copper grids. Samples were viewed with a Zeiss 10C TEM at 80 keV. Micrographs were collected on Kodak 4489 film, processed, and digitized using an Agfa Duoscan flatbed scanner.

Metal analyses

Soluble fractions from *E. coli* cell lysates and different growth media were analyzed in triplicate for Zn(II) content by using a Varian Liberty 150 ICP-AES. The emission wavelength was set at 213.856 nm for Zn(II), and a calibration curve with four standards and a correlation coefficient of greater than 0.999 was generated and used to determine Zn(II) levels in the experimental samples.

Acetone precipitation and sample cleanup for 2D gels

The sample (not less than 0.1 mg/ml) to be precipitated was mixed with an equal volume of prechilled acetone at −20 °C and was vortexed. The mixture was incubated on ice for 30 min and was centrifuged at top speed in a refrigerated microcentrifuge for 30 min. The pellet was dried and resuspended thoroughly in isoelectric focusing (IEF) sample buffer containing 8 M urea, 50 mM dithiothreitol, 4% CHAPS, 0.2% carrier ampholytes, and 0.0002% bromophenol blue. A 500 μg sample was used in a gel cleanup procedure using BioRad's ReadyPrep 2-D cleanup kit.

IEF and second dimension SDS-PAGE

Protein samples (100–300 μg) were dissolved in rehydration/sample buffer containing 8 M urea, 50 mM dithiothreitol, 4% CHAPS, 0.2% carrier ampholytes, and 0.0002% bromophenol blue. Isoelectric focusing was conducted on 11-cm IPG strips (pH 4–7) by using a Protean IEF Cell (Bio-Rad). These samples required 80,000–100,000 volt-hours for optimum focusing. After focusing, the IPG strips were stored at −80 °C until used in subsequent steps. Reduction and alkylation of cysteines were conducted by treating the strips in equilibration buffer I (6 M urea, 2% SDS, 0.05 M Tris–HCl, pH 8.8, 20% glycerol, and 2% dithiothreitol) and equilibration buffer II (6 M urea, 2% SDS, 0.05 M Tris–HCl, pH 8.8, 20% glycerol, and 2.5% iodoacetamide). The second dimension SDS-PAGE gel was run by using Criterion Precast gels (Tris–HCl, 8–16% resolving, 4% stacking), and the gels were stained with Coomassie blue.

Imaging and image analysis

Images of the gels were collected by using the Versa Doc 1000 Imaging System (Bio-Rad) and analyzed by using the PDQuest 2-D Analysis Software 7.1.1 (BioRad). The number of protein spots was ascertained by using the Spot Detection wizard in the PDQuest software package. Match sets were generated from replicate gels to compare the corresponding protein spots in two gels obtained from the test and control conditions. In order to make two gels quantitatively comparable, the images were normalized to account for differences in staining intensities, imaging, and sample loading variabilities.

In-gel trypsin digestions, MALDI-MS analyses, and database searching

Protein spots were excised and vortexed with 100 μl of 25 mM NH_4HCO_3/50% acetonitrile solutions for 10 min. This step was repeated at least twice before drying the gels completely by using a Speed Vac (Savant Instruments, Holbrook, NY). The dried gel pieces were then treated with 25 μl of 10 mM dithiothreitol in 25 mM NH_4HCO_3 and incubated at 56 °C for 1 h. The supernatant was removed, 25 μl of 55 mM iodoacetamide was added onto the gels, and the mixture was incubated at room temperature for 45 min in the dark. The supernatants were discarded, the dried gel pieces were treated with 25 μl of 12.5 ng/$l trypsin in 25 mM NH_4HCO_3, and the mixtures were incubated overnight at 37 °C. The supernatants from the trypsin-digested mixtures were collected in separate tubes, and peptides were extracted twice by treating the gel pieces with 30 μl 50% acetonitrile/5% formic acid and centrifuging the samples for 5 min. The extract was dried to a 10 μl volume by using a Speed Vac. A 2 μl sample of the peptide extract was mixed with 2 μl of 10 mg/ml cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.05% trifluoroacetic acid, and 2 μl of this mixture was loaded onto a matrix-assisted laser desorption/ionization (MALDI) target. A Bruker Reflex III MALDI-MS instrument was employed for MALDI-time-of-flight (TOF)-MS analysis of trypsin-digested peptides. The instrument was calibrated by using angiotensin II (human) with an m/z value of 1046.5423 and ACTH fragment 18–39 (human) with an m/z value of 2465.1989. Both of the standard peptides were obtained from Sigma. Mass spectra were obtained by setting the instrument in reflectron mode with reflectron detector voltage of 1.45 kV. Spectra were collected in the m/z range of 1000–2600 Da.

Peptide mass fingerprints obtained by MALDI-TOF analyses were used to search *E. coli* databases in SWISS-PROT (http://us.expasy.org) and NCBI-GenBank (http://www.ncbi.nlm.nih.gov), and protein identifications were made by using ProFound (http://prowl.rockefeller.edu) and MASCOT (http://www.matrixscience.com). The parameters used for the searches were as follows: variable modifications were considered (cysteine as the *S*-carboamidomethyl derivative and methionine in oxidized form); up to two missed cleavage sites were allowed; and restriction was placed on p*I* range 4–7. At least five peptides, representing a minimum of 20% sequence coverage, were considered as the minimum criteria for protein identity.

Results

Effect of Zn(II) on E. coli cell growth and morphology

In order to probe for Zn(II) responsiveness, *E. coli* were cultured in minimal media with no added

Zn(II) (mm-Zn) or in minimal media containing 250 μM added Zn(II) (mm + Zn), and growth curves were obtained (Figure 1). Both cultures reached saturation at 7.5 h, and the similar growth curves suggest that any changes in protein expression can be attributed directly to differing Zn(II) concentrations in the media rather than to stress caused by differing growth media, Zn(II) toxicity, etc. The addition of Zn(II) at concentrations higher than 250 μM to the minimal medium resulted in lower growth rates for *E. coli*. Transmission electron microscopy images of *E. coli* cells cultured in mm-Zn and mm + Zn media demonstrated no discernable differences in these cells (Figure 2); therefore, *E. coli* cultures grown in mm-Zn and mm + Zn were analyzed with 2D gel electrophoresis (see below).

To determine the Zn(II) concentrations in mm-Zn and mm + Zn before and after cell growth, metal analyses were conducted. Before cell growth, mm-Zn and mm + Zn media contained 0.09 and 13 μmoles Zn(II) (in 50 ml), respectively. It was surprising that mm-Zn contained so much Zn(II) as the medium was treated with Chelex-100, and all glassware was acid-washed before being used in these studies. After *E. coli* cell growth, the cells were collected by centrifugation, and the supernatants from cultures grown in mm-Zn and mm + Zn were analyzed for metal content and shown to contain 0.08 μmoles and 1.7 μmoles (in 50 ml), respectively. The cell pellets from the cultures were washed twice with 10 ml of 30 mM Tris, pH 7.5, containing 200 mM NaCl and lysed, and the cell debris was removed by centrifugation, resulting in fractions containing soluble proteins. These soluble fractions from the mm-Zn and mm + Zn cultures were shown to contain 0.01

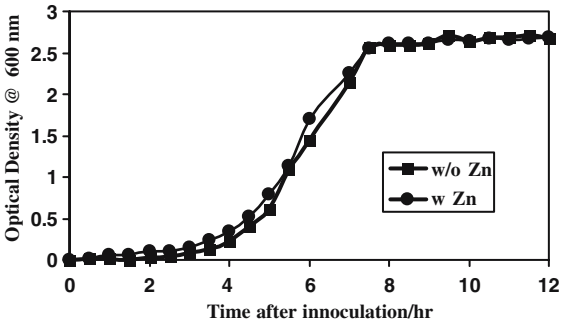

Figure 1. Growth curves for *E coli* grown in minimal media with no added Zn(II) (■) and with 250 μM added Zn(II) (●).

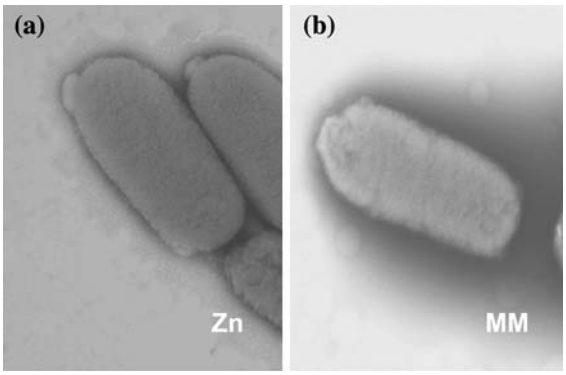

Figure 2. Transmission electron microscopy (TEM) images: *E. coli* cells were negative stained with 1.5% (w/v) ammonium molybdate on carbon coated, collodion substrated 300 mesh copper grids. Samples were viewed with a Zeiss 10C TEM at 80 keV. Cells grown in (a) minimal medium with 250 μM Zn(II) (b) minimal medium with no added Zn(II).

μmoles and 0.21 μmoles Zn(II) (in 10 ml), respectively. In the mm + Zn cultures, most of the initial Zn(II) probably precipitates as $Zn_3(PO_4)_2$ or nonspecifically binds to lipids and insoluble proteins that are removed during centrifugation. Nonetheless, these results demonstrate that the intracellular concentrations of Zn(II) can be affected by the extracellular concentrations of Zn(II).

2D gels of E. coli cultures

The soluble fractions of *E. coli* cells cultured in mm-Zn and mm + Zn were analyzed by 2D gel electrophoresis. A total of 258 protein spots could be resolved by using 11 cm immobilized pH gradient (IPG) strips (pH 4–7) (data not shown). In an effort to increase the number of resolved protein spots, the soluble proteins were further fractionated by using DEAE Sepharose chromatography as previously described (Sigdel *et al*. 2004). Representative 1D SDS-PAGE gels of the DEAE Sepharose column fractions are shown in Figures 3 and 4.

The DEAE-Sepharose fractions of multiple cultures grown in mm + Zn and mm-Zn were subjected to 2D gel electrophoresis. These gels were analyzed with PDQuest, and any protein spots that exhibited 1.5-fold or more change in density between the two matching set of gels were considered as proteins of interest. A total of 429 protein spots were excised from the 2D gels and subjected to in-gel trypsin treatments followed by

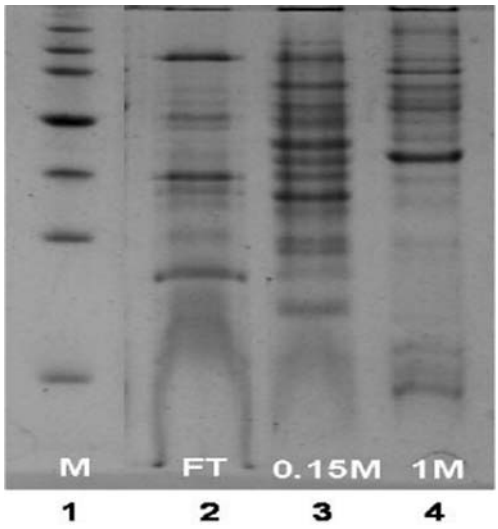

Figure 3. Fractionation of soluble *E. coli* proteins cultured in mm + Zn by DEAE Sepharose chromatography. SDS-PAGE gel of fractions: lane 1: molecular weight markers; lane 2: proteins that did not bind to column (flow through); lane 3: proteins that eluted from the column at 0.15 M NaCl; and lane 4: proteins that eluted with 1 M NaCl.

MALDI-MS analyses and database analyses. A total of 218 proteins were identified by using this approach.

Twelve proteins exhibited significant, reproducible increased expression levels as a result of elevated Zn(II) levels (Table 1). All of these proteins are involved in various metabolic processes, and none have previously been associated with Zn(II). Outer membrane proteins, OmpA and OmpF, constitute a two member porin family and are known to be involved in nonspecific transport of small molecules <600 Da (Sugawara & Nikaido 1992). OmpA has been reported to be essential in stress conditions as a deletion-mutant line of OmpA was found to be more sensitive towards stress due to SDS, cholate, highly acidic environments, etc. (Wang 2002). Although OmpA and OmpF are membrane-spanning proteins and thought to be insoluble, these proteins have been observed in previous 2D gels (Yohannes *et al.* 2004) and are probably at least partially soluble. ArtI, which is part of the ArtPMQJI arginine/ABC transporter system (Linton & Higgins 1998), shares sequence homology with other periplasmic ABC transporters; however, the substrate for ArtI is still unknown (Wissenbach *et al.* 1995; Linton & Higgins 1998). The levels of ArtI were found to be increased by acetate stress in a separate study (Kirkpatrick *et al.* 2001). The glycolytic enzymes enolase (Eno) and GpmA were found to be expressed highly in Zn(II) added medium. Eno, which requires Mg(II) for its enzymatic activity (Spring & Wold 1971), has been reported to be a member of the RNA degradosome complex (Liou *et al.* 2001). Phosphoglycerate mutase (GpmA) has recently been found to be regulated by ferric uptake regulator (Fur), which has been predicted to be involved in the regulation of general metabolic pathways (Vassinova & Kozyrev 2000). Interestingly in *Pasteurella multocida*, the genes for a Zn(II) transport system are thought to regulated by Fur and Fe (Garrido *et al.* 2003). This result along with the fact that Fur is a Zn(II) binding protein (Althaus *et al.* 1999) suggests that there may be a link in Zn(II) and iron homeostasis. CysK and GlyA are enzymes involved in amino acid metabolism. Stancik *et al.* (2002) have reported an up-regulation in the expression of CysK in *E. coli* stressed by high pH (Stamper *et al.* 2001) and cold shock (Graumann *et al.* 1996). GroEL and trigger factor represent two out of the four intracellular molecular chaperones in *E. coli* (Houry 2001). Trigger factor has been reported to be regulated by Cd(II) stress (Ferianc *et al.* 1998), and a homolog in *Thermus thermophilus* (Suno *et al.* 2004) has recently been reported to bind Zn(II). Lastly, the most abundant protein in *E. coli*, TufB (Weijland *et al.* 1992), was upregulated along with the ribosomal protein L9 (Herbst *et al.* 1994) and carbamoyl phosphate synthase (CarB) (Rubino *et al.* 1987).

Nine proteins exhibited significant, reproducible decreased expression levels as a result of elevated Zn(II) levels (Table 2). All of these proteins are also involved in various metabolic processes, and no link to Zn(II) has been reported for any of them. Mn-containing superoxide dismutase (Mn) (SodA) has been reported to regulate glycerol metabolism by controlling the activity of glycerol kinase (Benov & Al-Ibraheem 2001) in addition to its role in the detoxification of reactive oxygen species (O_2^-) (Hopkin *et al.* 1992). Outer membrane protein X (OmpX), which was reported to be induced either by low and high pH (Stancik *et al.* 2002), is involved in cell-surface interactions, and its homologs in other organisms have been implicated in bacterial virulence (Mecsas *et al.* 1995). ProX, the periplasmic component of the ABC transporter responsible for high-affinity

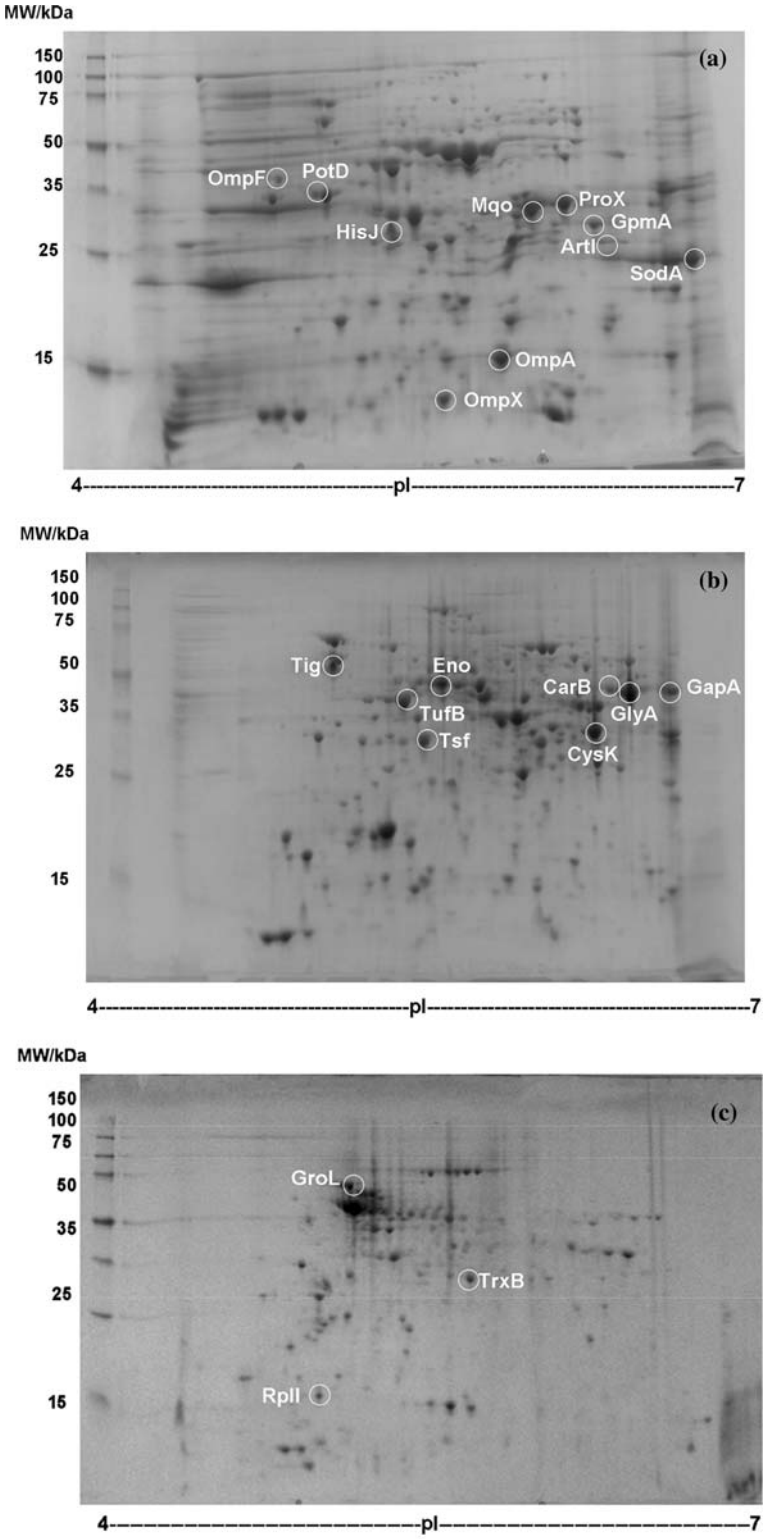

Figure 4. Gel images of 2D gels of the fractions obtained from the DEAE sepharose column with proteins IDs obtained for *E. coli* cells grown in the minimal medium with supplemented 250 μM $ZnSO_4$. (a) 2D gel image of the flow-through fraction with protein IDs on the gel; (b) 2D gel image of the fraction eluted with the elution buffer containing 0.15 M NaCl with protein IDs labeled; and (c) 2D gel image of the fraction with the elution buffer containing 1 M NaCl with protein IDs labeled.

Table 1. List of proteins whose expression level was found to be increased in the presence of 250 μM added Zn(II) in the minimal medium.

	Blattner #	Protein ID	Size/kDa	Fold increase	Function
1	B0957	Outer membrane protein 3a (OmpA)	37	1.9	Outer membrane porin, nonspecific diffusion channel (Sugawara & Nikaido 1994)
2	B0929	Outer membrane porin (OmpF)	39	1.9	Membrane porin reported to be down regulated in presence of high solute (Nikaido 1992)
3	B0863	Arginine 3rd transport system periplasmic binding protein (ArtI)	27	1.8	High affinity arginine binding and transporting (periplasmic) (Wissenbach *et al.* 1995)
4	B2779	Enolase (Eno)	46	2.0	Energy metabolism, glycolysis (Gerlt *et al.* 2005)
5	B0755	Phosphoglyceromutase (GpmA)	29	1.6	Energy metabolism, glycolysis (Bond *et al.* 2002)
6	B2414	Cysteine Synthase A (CysK)	34	5.9	Aminoacid biosynthesis, cysteine (Weijland *et al.* 1992; Quadroni *et al.* 1996)
7	B4143	GroEL, chaperone HSP 60 (GroL)	57	2.2	Chaperone, heat shock protein (Horwich *et al.* 1993)
8	B3980	Chain C, Ef-Tu/Ef-Ts (TufB)	43	1.8	Protein translation and modification, cytoplasm, osmotic stress response (Weijland *et al.* 1992)
9	B3986	Ribosomal protein L9 (RplI)	16	10[a]	Protein biosynthesis, component of ribosome (Herbst *et al.* 1994)
10	B2551	Serine hydroxymethyltransferase (GlyA)	45	8.3	Amino acid biosynthesis, glycine (Schirch *et al.* 1985)
11	B0033	Carbamoyl phosphate synthase (CarB)	117	1.9	Ribonucleotide biosynthesis, pyrimidine nucleotide biosynthesis (Rubino *et al.* 1987)
12	B0436	Trigger factor (Tig)	48	1.9	Chaperone related to cell division, protein folding (Scholz *et al.* 1997)

[a]Spot was not detected on matching gel.

transport of osmoprotectant glycine betaine (Wu *et al.* 1995), was reported to be up-regulated with cold shock (Polissi *et al.* 2003) and down-regulated with acetate and formate stress (Kirkpatrick *et al.* 2001; Polissi *et al.* 2003). HisJ is the periplasmic component of the ABC transporter responsible for histidine transport (Liu & Ames 1998). GapA is the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Doolittle *et al.* 1990), and the expression of this enzyme has been reported to be heat induced (Riehle *et al.* 2003). Tsf is part of elongation factor Ts (EF-Ts) that is involved with translation and has recently been reported to bind Zn(II) (Kawashima *et al.* 1996; Katayama *et al.* 2002). Expression of the EF-Ts gene has been reported to be induced by acidic pH of the growth medium (Birch *et al.* 2003). MqO is the membrane-associated malate dehydrogenase that catalyzes the essentially irreversible oxidation of malate to oxaloacetate (van der Rest *et al.* 2000). PotD is the periplasmic substrate-binding protein that recognizes and facilitates the transport of polyamines (Kashiwagi *et al.* 1993). TrxB is the NADPH-dependent thioredoxin reductase, which reduces disulfides in proteins (Kern *et al.* 2003). This protein has been reported to prevent aggregation of citrate synthase under heat-shock conditions and has been suggested to have chaperone-like function (Kern *et al.* 2003).

Discussion

There have been attempts to study changes in protein levels in *E. coli* in response to different stimuli using proteomics approaches. For example,

Table 2. A list of proteins whose expression level was found to be decreased in the presence of 250 μM added Zn(II) in the minimal medium.

	Blattner #	Protein ID	Size	Fold decrease	Function
1	B3908	Superoxide dismutase(Mn) (SodA)	23	2.2	Removal of superoxide radicals (Fee 1991)
2	B0814	Outer membrane protein (OmpX)	19	2.5	Outer membrane porin function not clear...may be cation transport (Mecsas *et al.* 1995)
3	B2679	Glycine betaine-binding periplasmic protein (ProX)	36	1.7	Periplasmic component of high-affinity transport system for glycine, betaine, and proline (Barron *et al.* 1987)
4	B2309	Component of His binding ABC transporter (HisJ)	28	2.5	High affinity his binding and transporting protein of ABC cassette (periplasmic) (Wu *et al.* 1995)
5	B0170	Chain D, Ef-Tu/Ef-Ts (Tsf)	30	2.0	Protein translation and modification (Zhang *et al.* 1998)
6	B2210	Malate dehydrogenase (Mqo)	60	2.2	TCA cycle oxidation of malate to oxaloacetate, membrane (cytoplasm mdh gene does the same) (van der Rest *et al.* 2000)
7	B1123	Spermidine putrescine-binding protein (PotD)	39	2.0	Polyamine binding periplasmic member of ABC transporter (Kashiwagi *et al.* 1993)
8	B1779	Glyceraldehyde-3-phosphate dehydrogenase (GapA)	40	5.0	Energy metabolism, glycolysis (Riehle *et al.* 2003)
9	B0436	Thioredoxin reductase (TrxB)	35	2.0	2'-deoxyribonucleotide metabolism (Williams 1995)

Kirkpatrick *et al.* (2001) demonstrated that the expression levels of several *E. coli* proteins were affected by extracellular concentrations of acetate and formate. By using 2D gels and protein identifications, Lambert *et al.* (1997) reported that many *E. coli* proteins are up-regulated by the presence of benzoic acid in the growth medium. Ferianc *et al.* (1998) utilized 2D gels to identify cadmium induced proteins in *E. coli*, and most of the identified proteins were stress-related proteins. Two-dimensional gel electrophoresis/peptide identifications have become a popular tool to study changes in protein expression levels in response to external stimuli (Zhou *et al.* 2004; Poon *et al.* 2005; Snijders *et al.* 2005).

In this context, we employed proteomics to identify the proteins in *E. coli* whose expression levels changed in response to differing concentrations of extracellular Zn(II). Metal analyses of the culture media demonstrated that the levels of intracellular Zn(II) could be affected by varying the concentrations of extracellular Zn(II). In addition, growth curves and microscopy demonstrated that the cells used in our protocol are phenotypically similar. Zn(II)-responsive proteins were detected in 2D gels and were identified by MALDI-TOF MS analyses and database searching. Surprisingly, none of the proteins identified with our studies have previously be implicated in Zn(II) homeostasis (Table 3), and none of the Zur-regulated proteins (YkgM, ZnuABC, YodA) exhibited differential expression levels. It is possible that the levels of these proteins may not have been high enough for detection by using 2D gel electrophoresis/protein identifications or that positive identifications of these proteins from excised spots on the gels could not be made. We purposely chose the IPG strips of pI's of 4–7 because all of the proteins thought to be involved with Zn(II) transport in *E. coli* (ZnuA, ZitB, Zur, ZupT, and ZntR) have predicted pI values between 4 and 7. It may also be possible that cellular response to differential Zn(II) levels is rapid, and the procedure used in this study identified adaptive response changes. None of the identified proteins in our study have previously been associated with Zn(II); however, many of the proteins are involved with metabolic processes. This result supports the previously predicted role of Zn(II) in the modulation of other proteins in *E. coli* (Vallee 1988; Coleman

Table 3. Summary of the impact of Zn(II) in the growth medium in different cell activities.

Protein category	Protein IDs
Detoxification proteins	SodA
Outer membrane proteins	OmpX, OmpF, OmpA
Glycolysis and TCA cycle	Eno, GapA, Mqo, GpmA
Periplasmic members of ABC transporters	PotD, ArtI, HisJ, ProX
Chaperones/heat shock proteins	Tig, GroL
Ribosome/Translation related	TufB, Tsf, RplI
Amino acid biosynthesis	GlyA, CysK
Ribonucleotide biosynthesis/ deoxynucleotide metabolism	CarB, TrxB

1992; Gaither & Eide 2001; Hantke 2001; Blencowe & Morby 2003; Chimienti *et al*. 2003; Liuzzi & Cousins 2004; Maret 2004).

Brocklehurst and Morby (2000) reported a number of genes that were regulated by metal tolerant *E. coli*. By using a whole-genome transcriptional analysis of these cells, *ompA* and*tufB* genes, along with 18 other genes, were shown to be down-regulated by excess Zn(II). Lee *et al*. (2005) reported a global response on the transcriptome of *E. coli* due to the presence of 0.2 mM Zn(II) in chemostat cultures. A number of genes were shown to be regulated by high Zn(II) concentrations in the medium, and most of the gene products were stress related and not previously believed to be associated with Zn(II). Out of a total 122 genes that were reported to be regulated by elevated Zn(II) by Lee *et al*. (2005), only one gene product, CysK, overlapped with the proteins identified with our proteomic analyses. This surprising result is most likely due to the relatively lower detection limits of the 2D gel electrophoresis based technique; however, it is possible that transcript levels in *E. coli* do not directly correlate directly with protein levels. It is not clear why the DNA arrays did not detect the proteins identified in our proteomic studies; however, Lee *et al*. (2005) stressed *E. coli* cells with Zn(II) excess in their study, while our experiments were designed to probe Zn(II) responsiveness in non-stress conditions.

Studies are currently underway to probe for the rapid and adaptive response of *E. coli* to stress due to Zn(II) excess and deficiency by using proteomic and genomic approaches.

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