

Quantitative proteomic profiling of the *Escherichia coli* response to metallic copper surfaces

Renu Nandakumar · Christophe Espirito Santo ·
Nandakumar Madayiputhiya · Gregor Grass

Received: 28 December 2010 / Accepted: 24 February 2011 / Published online: 8 March 2011
© Springer Science+Business Media, LLC. 2011

Abstract Metallic copper surfaces have strong antimicrobial properties and kill bacteria, such as *Escherichia coli*, within minutes in a process called contact killing. These bacteria are exposed to acute copper stress under dry conditions which is different from chronic copper stress in growing liquid cultures. Currently, the physiological changes of *E. coli* during the acute contact killing process are largely unknown. Here, a label-free, quantitative proteomic approach was employed to identify the differential proteome profiles of *E. coli* cells after sub-lethal and lethal exposure to dry metallic copper. Of the 509 proteins identified, 110 proteins were differentially expressed after sub-lethal exposure, whereas 136 proteins had

significant differences in their abundance levels after lethal exposure to copper compared to unexposed cells. A total of 210 proteins were identified only in copper-responsive proteomes. Copper surface stress coincided with increased abundance of proteins involved in secondary metabolite biosynthesis, transport and catabolism, including efflux proteins and multidrug resistance proteins. Proteins involved in translation, ribosomal structure and biogenesis functions were down-regulated after contact to metallic copper. The set of changes invoked by copper surface-exposure was diverse without a clear connection to copper ion stress but was different from that caused by exposure to stainless steel. Oxidative posttranslational modifications of proteins were observed in cells exposed to copper but also from stainless steel surfaces. However, proteins from copper stressed cells exhibited a higher degree of oxidative proline and threonine modifications.

Electronic supplementary material The online version of this article (doi:10.1007/s10534-011-9434-5) contains supplementary material, which is available to authorized users.

R. Nandakumar · N. Madayiputhiya
Proteomic and Metabolomic Core Facility, Redox
Biology Center, Department of Biochemistry, University
of Nebraska-Lincoln, Lincoln, NE 68588, USA

C. Espirito Santo
Department of Life Sciences, Faculty of Sciences and
Technology, University of Coimbra and Marine and
Environmental Research Center (IMAR-CMA),
3001-401 Coimbra, Portugal

C. Espirito Santo · G. Grass (✉)
School of Biological Sciences, University of Nebraska-
Lincoln, Beadle Center, E141, Lincoln, NE 68588, USA
e-mail: ggrass2@unl.edu

Keywords Copper surfaces · *Escherichia coli* ·
Quantitative proteomics · Posttranslational
modification

Introduction

Metallic copper surfaces have powerful antimicrobial properties. Most microbes exposed to copper surfaces are killed within minutes to a few hours. This fast killing applies to Gram-positive and -negative

bacteria (Molteni et al. 2010; Noyce et al. 2006; Mehtar et al. 2007; Elguindi et al. 2011), single-celled yeasts such as *Saccharomyces* and *Candida*, as well as the spores of some multicellular ascomycetes (Quaranta et al. 2011; Weaver et al. 2010). Recently, factors that lead to killing of copper surface-exposed cells have been elucidated and major sensitive targets of toxicity in the cell were investigated (Espirito Santo et al. 2011). Contact to dry metallic copper surfaces result in the accumulation of molar concentrations of copper ions in *E. coli*. These copper ions are dissolved from the metallic copper within a few seconds and quickly overwhelm the cellular copper defense systems such as the CusCFBA and CopA Cu(I)-efflux systems and the multicopper oxidase CueO (Espirito Santo et al. 2008). This provided a rationale for the limited contribution of these systems for survival on metallic copper (Elguindi et al. 2009; Molteni et al. 2010). Additionally, exposure to metallic copper also causes extensive oxidative stress in cells (Quaranta et al. 2011; Espirito Santo et al. 2011), probably connected to copper-mediated Fenton-like reactions. While genotoxicity caused by metallic copper remains a controversial subject (Warnes et al. 2010), multiple lines of evidence suggests that cells are killed through lethal membrane damage in pro- and eukaryotes (Quaranta et al. 2011; Espirito Santo et al. 2011). Our current knowledge indicates that there is a great overlap between copper ion toxicity and toxicity mediated by contact to metallic copper. However, significant differences exist. Cells on dry metallic copper are acutely (seconds up to few hours) stressed and do not grow in this environment. Chronically exposed cells are stressed with copper ions in their growth media supplemented with toxic copper concentrations and are able to proliferate under these conditions. As such, chronically exposed growing cells are usually faced with low millimolar copper ion concentrations, whereas those acutely exposed on surfaces have to deal with low molar concentrations of dissolved copper ions.

We are well informed about the physiological responses of growing bacterial cells challenged with various concentrations of copper ions. Both transcriptomic (Yamamoto et al. 2005; Kershaw et al. 2005; Teitzel et al. 2006) and proteomic studies (Magnani et al. 2008; Miller et al. 2009; Sharma et al. 2006; Monchy et al. 2006) have addressed the gene expression response after chronic copper exposure.

However, quantitative proteomic analysis either by labeled or label-free methods have been used only rarely for the elucidation of cellular defenses and stress response mechanisms against copper (Bagwell et al. 2010; Kao et al. 2004). Similar studies with acute copper surface-exposed cells are lacking. Global, quantitative proteome profiling based on multidimensional shotgun approaches have so far not been applied to gain comprehensive insight into the cellular responses in *E. coli* on exposure to copper surfaces. Such studies would provide insights into the last minutes of the lives of these lethally stressed bacteria and can be expected to be the starting point for further genetic and physiological studies addressing sensitive cellular targets of severe copper toxicity and potential emerging resistance mechanisms.

In this study we investigated the global protein expression profile of *E. coli* cells acutely and lethally stressed by exposure to metallic copper surfaces and compared to the proteome of cells after contact to stainless steel.

Materials and methods

Growth conditions and cell exposure

The strain used in this study was *Escherichia coli* K12 derivative W3110. *E. coli* was grown in Luria–Bertani (LB) broth (Difco BD), at 37°C for 16 h with rotary shaking (250 rotations per minute, RPM). Cells were concentrated with phosphate buffer saline (PBS), applied to a cotton swab, spread on a metal surface and incubated at room temperature for a few seconds (needed to let the surface completely dry) or for 5 additional min. The former will be referred to throughout this study as sub-lethal and 5 min as lethal exposure time, a time after which cells are no longer able to develop into colonies (Espirito Santo et al. 2008). Three biological replicates were included for each condition that was studied. Metal surfaces were 2.5 × 2.5 cm copper coupons (C11000, 99.9% copper) and stainless steel control coupons (AISI 304, approx. 67–72% Fe, 17–19.5% Cr, 8–10.5% Ni). All copper-alloy coupons were disinfected and cleaned prior to each experiment to standardize the surface properties as described previously (Espirito Santo et al. 2008). Cells were resuspended and removed from copper or stainless steel surfaces with 50 µl of

50 mM ammonium bicarbonate containing 8 M urea and transferred into an ice-cold centrifuge tube and the cells were harvested by centrifugation.

Sample preparation

Cells were resuspended in 50 mM ammonium bicarbonate containing 8 M urea and 1.5 mM phenylmethylsulfonyl fluoride (PMSF) and lysed with glass beads for 2.5 min, on ice, using a miniBead Beater (BioSpec Products) (Nägele et al. 2003). Cell lysates were centrifuged ($16,000\times g$, 10 min at 4°C) to remove the cell debris and glass beads. The bacterial proteins in the supernatant were precipitated by acetone and the resultant protein pellets were resuspended in 100 mM ammonium bicarbonate containing 6 M urea. Protein concentrations were determined using the 2-D Quant kit (GE Health Care). The proteins were further reduced with 10 mM dithiothreitol and alkylated with 40 mM iodoacetamide. The samples were buffer exchanged with 50 mM ammonium bicarbonate and digested with sequencing-grade trypsin (Roche) (1:50 trypsin: protein ratio) overnight at 37°C (Nandakumar et al. 2009). Following proteolytic digestion, samples were desalted and concentrated by solid phase extraction (PepClean C-18 spin columns, Pierce), vacuum-dried and stored at -80°C prior to 2DLC-MS/MS analysis.

2DLC-MS/MS analysis

2DLC-MS/MS analysis was performed using an Ultimate 3000 Dionex MDLC system (Dionex Corporation, CA) integrated with a nanospray source and LCQ Fleet Ion Trap mass spectrometer (ThermoFinnigan, USA). Initially, a first dimension LC separation [strong cation-exchange (SCX) chromatography] with fraction collection was performed followed by the second dimension LC separation (reverse phase chromatography) and subsequent detection by tandem mass spectrometry. The first dimensional separation was performed on a SCX column (Polysulfoethyl, 1 mm I.D \times 15 cm, 5 μm , 300A, Dionex). 20 μl of sample was loaded onto first dimension SCX column and eluted using a salt gradient of 0–600 mM. Selected fractions based on the UV absorbance of the eluted peptides were subjected to second dimension analysis. The second dimension

separation included an on-line sample pre-concentration and desalting using a monolithic C 18 trap column (Pep Map, 300 μm I.D \times 5 mm, 5 μm , 100A, Dionex). The loading of the sample on the monolithic trap column was done at a flow rate of 20 $\mu\text{l}/\text{min}$. The desalted peptides was then eluted and separated on a C 18 Pep Map column (75 μm I.D \times 15 cm, 3 μm , 100 A) applying an acetonitrile (ACN) gradient (ACN plus 0.1% formic acid, 90 min gradient) at a flow rate of 300 nl/min using a micro pump and introduced into the mass spectrometer using the nano spray source. The LCQ Fleet mass spectrometer was operated with the following parameters: nano spray voltage 2.0 kV, heated capillary temperature 200°C and full scan m/z range 400–2,000. The LCQ was operated in data dependent mode with 4 MS/MS spectra for every full scan, 5 microscan averaged for full scans and ms/ms scans, 3 m/z isolation width for ms/ms isolations and 35% collision energy for collision induced dissociation. Dynamic exclusion was enabled with exclusion duration of 1 min.

Proteome bioinformatics and data analysis

The acquired MS/MS spectra were searched against the *E. coli* K12 database (4,149 sequences) using MASCOT (Version 2.2 Matrix Science, UK) with the following parameters: enzyme: trypsin; missed cleavages: 2; mass: monoisotopic; fixed modification: carbamidomethyl (C); peptide tolerance: 1.5 Da; MS/MS fragment ion tolerance: 1 Da. Probability assessment of peptide assignments and protein identifications were accomplished by Scaffold (Proteome Software Inc., Portland, OR). Only peptides with $\geq 90\%$ probability were considered. Criteria for protein identification included detection of at least two unique peptides and a protein probability score of $\geq 90\%$. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Relative quantitation of the proteins was done using the label-free method of spectral counting (Liu et al. 2004) using the normalized spectral counts for each protein. A P -value ≤ 0.05 (Fisher's exact test) combined with a 2-fold change in abundance were used to select for proteins that were differentially expressed between the proteomes under comparison.

Additional database searches were performed using MASCOT specifying the following posttranslational modifications as variable modifications: oxidation of lysine to amino adipic semialdehyde (−1 Da), arginine to glutamic semialdehyde (−43 Da), proline to 2-pyrrolidone (−28 Da), threonine to 2-amino-3-ketobutyric acid (−2 Da), cysteine to cysteic acid (+48 Da), histidine to aspartic acid (−22 Da), oxidation of methionine, tyrosine and tryptophan (+16 Da) and the results were validated using the Scaffold software. For identification of proteins with posttranslational modifications, only peptides with $\geq 95\%$ probability were considered coupled with a 99% protein probability and two unique peptides per protein. All the spectra of the modified peptides were manually checked.

All identified proteins from our datasets were organized into COG functional category assignments using NCBI COGNITOR tool (Tatusov et al. 2000). The subcellular location of proteins from our datasets was predicted using PSORTb v3.0 (Gardy et al. 2005) and the number of transmembrane helices in membrane proteins were predicted by the TMHMM server 2.0 (Center for Biological Sequence Analysis, The Technical University of Denmark).

Results

Global proteome analysis

Protein abundance measurements were compared for *E. coli* cells sub-lethally and lethally shocked by exposure to metallic copper in order to assess the physiological responses during the killing process caused by this metallic copper challenge. The two time points (sub-lethal and lethal) were chosen to reflect different stages of the response to copper. An immediate short exposure would provide insight into the proteins produced or degraded as a consequence of the initial shock and the increase of copper ion and ROS concentration flooding the cell. Five minutes was used, because after 1 min cells have already accumulated lethal damage (Espirito Santo et al. 2008). Thus, we anticipated 5 min to be close to the endpoint status of cellular gene expression. Unchallenged cells served as negative controls. Cells exposed to stainless steel (same time periods as for copper) were used for comparison to differentiate

copper specific responses and those mediated by desiccation stress on the drying surfaces.

A total of 1,655 (40%) functionally diverse, non-redundant proteins with at least one unique peptide of high confidence out of the 4,149 predicted proteins were detected in *E. coli* control cells and cells exposed to copper. Of the 1,655 proteins, 509 were identified with at least two unique peptides and 169 proteins with three or more unique peptides. All the downstream biological interpretation is based on the proteins that were identified with two unique peptides and the entire list can be found in Table S1 (supplemental data). Further analysis indicated that 210 proteins were identified only in copper exposed cells and of them, 149 proteins were detected after exposing the cells to sub-lethal stress and the remaining 61 proteins were detected in cells exposed to copper for 5 min. In addition, 33 proteins identified after exposure to sub-lethal stress were not detected as the exposure time increased to 5 min.

Global functional distribution of the copper-responsive proteome

The functional distribution of the identified proteins in control cells and cells exposed to copper are shown in Fig. 1. The proteins in global datasets predominantly belonged to the functional categories of energy production and conversion (C), amino acid transport and metabolism (E) and carbohydrate transport and metabolism (G), with about 10% of the identified proteins in each dataset. Forty proteins (8%) did not belong to any of the currently defined COGs. In the unexposed proteome, proteins with functions in energy production and conversion (C), amino acid transport and metabolism (E), carbohydrate transport and metabolism (G) and translation, ribosomal structure and biogenesis (J) were predominant. The copper-exposed proteome datasets showed a higher proportion of proteins belonging to DNA replication, recombination and repair (L), cell envelope biogenesis (M) and signal transduction mechanisms (T) than control cells. The number of proteins with functional roles assigned to the categories of translation, ribosomal structure and biogenesis (J) and posttranslational modification, protein turnover, chaperones (O) was lower in the copper-exposed cells than the control cells. Poorly characterized proteins [those in the function unknown (S) category and those with

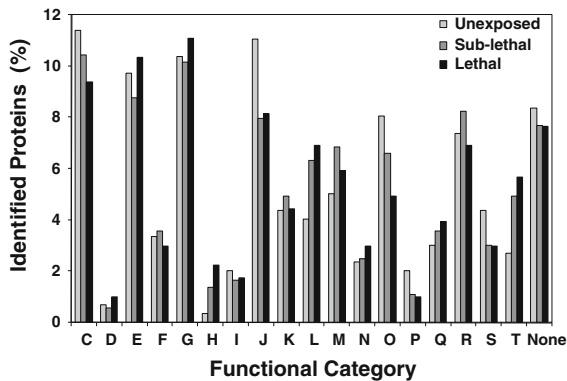


Fig. 1 Functional category distribution of proteins identified from *E. coli* cells exposed to elemental copper surfaces. Number of proteins belong to each functional category is shown as percentage of total identified proteins from each sample. COG categories are as follows: C: energy production and conversion; D: cell division and chromosome partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme metabolism; I: lipid metabolism; J: translation, ribosomal structure and biogenesis; K: transcription; L: DNA replication, recombination and repair; M: cell envelope biogenesis, outer membrane; N: cell motility and secretion; O: post translational modification, protein turnover, chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; R: general function prediction only; S: function unknown; T: signal transduction mechanisms; and None: no related COGs

general function prediction only (R)] and proteins with no related COGs constituted the largest proportion of the proteome datasets (20% in control cells and 17–19% in exposed cells). Proteins belonging to functional categories such as defense mechanisms (V) and intracellular trafficking, secretion and vesicular transport (U) were not identified in the proteome datasets.

All the enzymes involved in tricarboxylic acid (TCA) cycle were identified in the proteome datasets, except for fumarase (fumarate hydratase), with malate synthase A (b4014, AceB) being most prominent. The functional category of cell envelope biogenesis and outer membrane was very well represented with 31 proteins in addition to the six proteins involved in the inorganic ion transport and metabolism. Twenty-four proteins with assigned functions in the category of signal transduction mechanisms were identified, prominently 10 sensory histidine kinases in two-component regulatory systems, most of them up-regulated when exposed to copper. Proteins belonging to the functional category

of posttranslational modification, protein turnover and chaperones were highly represented in the global proteome profile of both the control and the exposed cells and also showed significant differential abundance between the proteome datasets. The most predominant were 10 well recognized chaperonins or those with chaperonin function as well as a peptidyl-prolyl cis/trans isomerase (b0436, Tig) that acts as a trigger factor. Also, the copper-responsive proteome was significantly represented by the functional category of secondary metabolite biosynthesis, transport and catabolism with many efflux proteins and multidrug resistance proteins.

Subcellular localization of identified proteins

To obtain information regarding the subcellular localization of the identified proteins, they were further analyzed by different algorithms. To predict the inner and outer membrane proteins, the PSORT algorithm was used. The analysis showed that the identified proteins are mainly localized in the cytoplasm and constituted the majority of the proteins identified (58%). Transmembrane helices in membrane proteins were predicted by the TMHMM server 2.0. Of the 97 proteins predicted to be of inner membrane origin, 46 proteins (47%) were predicted to contain two or more transmembrane domains. Thirty-seven proteins were sorted as periplasmic while nine each were of outer membrane or extracellular origin. Proteins of cytoplasmic membrane origin were more prevalent in the proteome of the cells exposed to copper than the control cells. Among them, proteins with functions in signal transduction mechanisms (13 proteins), carbohydrate transport and metabolism (13 proteins), energy production and conversion (12 proteins) were predominant and 24% of these proteins were transporters. The membrane proteins comprised efflux systems (b0462, AcrB; b3513, MdtE; b1879, FlhA and b3469, ZntA), a molecular chaperone of the HSP90 family (b0473, HtpG), sensory histidine kinases of two-component regulatory systems (b0619, Dpib; b3210, ArcB; b4125, DcuS; b0570, CusS; b2218, RcsC), as well as the uptake permeases b0760 (ModF), and b4111 (ProP). Amongst these was the sensor kinase CusS, needed for expression of the *cus* genes in response to copper ion stress.

Differential expression of proteins after sub-lethal exposure to copper

From our previous studies (Espirito Santo et al. 2008), it was established that exposure time plays an important role in copper surface inactivation of *E. coli* cells. Killing is extremely rapid, often just minutes, thus, impact of time of exposure on the protein expression levels was explored here. The quantitative proteomic profiles of *E. coli* unexposed cells and cells exposed to copper sub-lethally were compared to identify the proteins that showed significant differential expression (Table S2, supplemental data for the complete list). Table 1 lists the predominant proteins.

Comparative analysis of protein expression profiles of the unexposed *E. coli* cells and copper-exposed cells revealed a total of 110 proteins exhibiting statistically significant ($P \leq 0.05$) fold changes (≥ 2 -fold) in expression levels, with 50 proteins being up-regulated and 60 proteins down-regulated. In general, the most frequently represented COG functional groups in the up-regulated sub-proteome were carbohydrate transport and metabolism (five proteins), posttranslational modification, protein turnover, chaperones (five proteins), transcription (four proteins), amino acid transport and metabolism (three proteins), cell envelope biogenesis (three proteins), and signal transduction mechanisms (three proteins). Eleven proteins grouped under the functional category of DNA replication, recombination and repair were up-regulated, even though not statistically significant, after exposure to copper. Increased expression of DNA recombination and repair proteins, especially DNA polymerase III/DNA elongation factor III (b0470, DnaX) ($P = 5.5E-12$, 36-fold), which along with the up-regulation of chaperone Hsp70 (b0014, DnaK), ($P = 1.1E-08$, 2.5-fold) involved in intracellular protein-folding and stress responses preventing aggregation of stress denatured proteins, indicated the extent of toxic effects imposed by the copper ions in the cell environment. Another co-chaperone for DnaK, the chaperone GrpE (b2614) was also more abundant in exposed cells, however, not significantly.

Higher abundance was also observed for three proteins in the inorganic ion transport and metabolism category: superoxide dismutase (SodC, $P = 0.055$), bacterioferritin (b3336, Bfr, $P = 0.008$) and

hydroperoxidase (catalase, KatE, $P = 0.23$) that directly points to the possible cell response mechanisms to the copper challenge. All these enzymes have different roles in keeping reactants of Fenton-chemistry low. Superoxide dismutase and catalase decompose superoxide and hydrogen peroxide, respectively, and bacterioferritin oxidizes and stores superfluous ferrous iron for later use. The significant expression (6-fold) of SodC in response to sub-lethal copper exposure suggests an important role against copper toxicity. However, only one of the three superoxide dismutases of *E. coli*, SodC, was identified. Other proteins involved in general stress responses also exhibited significant up-regulation in copper-exposed cells. An osmotically inducible membrane protein, OsmC (b1482), is regarded as a stress-induced detoxification protein and was up-regulated (3-fold) after exposure to copper. In addition, peptides belonging to proteins such as predicted stress response protein (b4045, YjbJ) and a stress protein of the CspA-family (b1823, CspC) also exhibited increased abundance when cells were exposed to copper.

Two proteins grouped in the up-regulated signal transduction mechanisms category were sensory histidine kinases in two component regulatory systems involved in regulation of capsule biosynthesis (b2218, RcsC) and aerobic respiration control (b3210, ArcB). Further, transport proteins of sugar PTS systems (b2417, Crr; b4194, UlaB), a transpeptidase involved in peptidoglycan synthesis (b0084, FtsI), a SecYEG protein translocase auxiliary protein (b0407, YajC) and an acid-resistance protein (b3509, HdeB) were also among those up-regulated. Other prominent proteins with higher abundance included a putative amino acid (carbamate) kinase (b2874, YqeA), a predicted oxidoreductase with monooxygenase activity (b3690, CbrA), transketolase 1 (b2935, TktA), predicted protoheme IX synthesis protein (b3802, HemY) and an acyl carrier protein (b1094, AcpP). An outer membrane protein (b0957, OmpA) was significantly up-regulated (13-fold) after exposure to copper. OmpA is a porin with low permeability that allows slow penetration of small solutes. It is also worth noting that 22 proteins (44%) of the up-regulated sub-proteome were not detected in the control proteome dataset but were highly abundant in the copper-exposed proteome. Among these, proteins involved in amino acid transport and

Table 1 Predominant differentially expressed proteins after sub-lethal exposure to copper surfaces

Accession #	Gene	Description	Functional category	Fold change ^a
<i>Up-regulated after copper exposure</i>				
NP_417350	YqeA	Predicted amino acid kinase	E	40.3
NP_415003	DnaX	DNA polymerase III/DNA elongation factor III	L	36.1
NP_418145	CbrA	Predicted oxidoreductase with FAD/NAD(P)-binding domain	C	27.6
NP_415193	YbeZ	Predicted protein with nucleoside triphosphate hydrolase domain	T	18.0
YP_026188	TktA	Transketolase 1, thiamin-binding	G	13.8
NP_415477	OmpA	Outer membrane protein A	M	13.0
NP_418246	HemY	Predicted protoheme IX synthesis protein	H	12.7
NP_418615	UlaB	L-ascorbate-specific enzyme IIB component of PTS	G	12.7
NP_415403	ClpA	ATPase and subunit of ClpA-ClpP ATP-dependent serine protease	O	11.7
NP_415612	AcpP	Acyl carrier protein	I	11.1
NP_415810	SapA	Predicted antimicrobial peptide transporter	E	10.6
YP_026207	ArcB	Hybrid sensory histidine kinase in two-component regulatory system with ArcA	T	9.6
NP_414626	FtsI	Transpeptidase involved in septal peptidoglycan synthesis	M	8.5
NP_417877	MalT	DNA-binding transcriptional activator/maltotriose-ATP-binding protein	K	7.4
NP_414941	YajC	SecYEG protein translocase auxillary subunit	N	6.5
NP_416722	RcsC	Hybrid sensory kinase in two-component regulatory system with RcsB and YojN	T	5.3
NP_418469	YjbJ	Predicted stress response protein	S	4.8
NP_415999	OsmC	Osmotically inducible, stress-inducible membrane protein	O	3.0
NP_414555	DnaK	Chaperone Hsp70, co-chaperone with DnaJ	O	2.5
NP_417795	Bfr	Bacterioferritin, iron storage and detoxification protein	P	2.3
<i>Down-regulated after copper exposure</i>				
NP_415840	Tpx	Lipid hydroperoxide peroxidase	O	3.0
NP_418228	TrxA	Thioredoxin 1	O	3.8
NP_415138	AhpC	Alkyl hydroperoxide reductase	O	4.7
NP_417799	FusA	Protein chain elongation factor EF-G, GTP-binding	J	6.7
NP_416225	BtuE	Predicted glutathione peroxidase	O	8.0
NP_418567	GroL	Cpn60 chaperonin GroEL, large subunit of GroESL	O	8.7
NP_414660	AcnB	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	C	9.0
NP_417768	RplX	50S ribosomal subunit protein L24	J	9.0
NP_414859	YahK	Predicted oxidoreductase, Zn-dependent and NAD(P)-binding	R	9.4
NP_417083	ClpB	Protein disaggregation chaperone	O	11
NP_415257	SucD	Succinyl-CoA synthetase, NAD(P)-binding, alpha subunit	C	11
NP_418411	RplA	50S ribosomal subunit protein L1	J	12
NP_418366	HslU	Molecular chaperone and ATPase component of HslUV protease	O	12
NP_414712	Tsf	Protein chain elongation factor EF-Ts	J	13
NP_417172	LuxS	S-ribosylhomocysteinase	T	15
NP_417798	TufA	Protein chain elongation factor EF-Tu	J	18
NP_415006	HtpG	Molecular chaperone HSP90 family	O	24
NP_415268	TolB	Periplasmic protein	N	33
NP_418438	AceB	Malate synthase A	C	61
NP_418104	LigB	DNA ligase, NAD(+)-dependent	L	110

^a Compared to control cells

metabolism, energy production and conversion and cell envelope biogenesis were well represented, indicating the core areas of metabolism that actively respond to a short-term exposure to copper.

The most conspicuous feature of the copper-exposed proteome was the dramatic down-regulation of proteins involved in translation and in posttranslational modifications. The 60 proteins down-regulated after exposure to elemental copper constituted a subset dominated by proteins with annotated functions in translation, ribosomal structure and biogenesis (14 proteins), energy production and conversion (nine proteins) and posttranslational modification, protein turnover and chaperones

(eight proteins) illustrating the core metabolic processes affected by the presence of copper. The proteins that were affected dramatically by copper exposure were those with translation functions: protein chain elongation factor EF-Tu (b3339, TufA; b3340, FusA; b0170, Tsf), 30S and 50S ribosomal proteins (b4200, RpsF; b3303, RpsE; b3984, RplA; b3309, RplX; b3305, RplF) and those involved in posttranslational modifications: alkyl hydroperoxide reductase (b0605, AhpC, 5-fold), thioredoxin 1 (b3781, TrxA, 4-fold) and lipid hydroperoxide peroxidase (b1324, Tpx, 3-fold). The decreased expression levels of proteins involved in translation may comprise a general stringent response to stress or merely a function of lower metabolic rates in the presence of a toxic agent. In addition, expression levels of many chaperones such as Cpn60 chaperonin GroEL (b4143, GroL), protein-disaggregation chaperone (b2592, ClpB), molecular chaperone and ATPase (b3931, HslU), molecular chaperone HSP90 (b0473, HtpG) were also decreased after cells were exposed to copper. The periplasmic TolB protein (b0740) involved in uptake of group A colicins was prominent among the down-regulated proteins and was not identified in copper exposed cells. Many TCA cycle enzymes also showed significant down-regulation, especially malate synthase A (b4014, AceB, 61-fold). However, only one protein, *S*-ribosylhomocysteinase (b2687, LuxS), involved in signal transduction mechanisms was down-regulated (15-fold) and was in marked contrast to the up-regulation of numerous proteins (13 proteins) of the same functional category under identical conditions.

Eight proteins with no related COG categories were also differentially expressed after sub-lethal

exposure to copper with six proteins being up-regulated. A predicted protein (b5665, YgaU) putatively involved in cell wall macromolecule catabolic process was among those significantly down-regulated. A DNA ligase (b3647, LigB), which was present at a significantly higher level in the control proteome dataset, was not detected after exposure to copper. In fact, peptides attributed to 22 proteins which were identified in the control proteome dataset were no longer detected after copper exposure, dominated by those with assigned functions in energy production and conversion, as well as translation, ribosomal structure and biogenesis.

Differential expression of proteins after lethal exposure to copper

A total of 136 proteins exhibited a statistically significant altered abundance with at least a 2-fold change when the cells were exposed to elemental copper surfaces lethally for five min (Table S3, supplemental data) and the predominant proteins are listed in Table 2. After lethal exposure, 63 proteins were up-regulated and 73 were down-regulated with 34 proteins exhibiting drastic changes (≥ 10 -fold ratio) in their abundances. As the time of exposure increased from sub-lethal to lethal, the number of proteins in the down-regulated sub-proteome increased significantly.

In general, the up-regulated sub-proteome after five min constituted proteins grouping in the role of amino acid transport and metabolism (five proteins), energy production and conversion (nine proteins), cell envelope biogenesis (four proteins) and DNA replication, recombination and repair (four proteins). Among the up-regulated proteins, 12 proteins were expressed at levels higher after lethal stress than they were after sub-lethal stress. These comprised the maltose regulon positive regulatory protein (b3418, MalT), a putative carbamate kinase (b2874, YqeA), and a predicted CreB-regulated oxidoreductase with a FAD/NAD(P)-binding domain (b3690, CbrA). Biotin sulfoxide reductase (b3551, BisC) was detected at higher levels (12-fold) after lethal stress while it was not identified in the unexposed proteome and only in negligible levels after sub-lethal exposure. Eight of the differentially expressed proteins were detected only after lethal exposure and were not identified in the proteome of control cells or cells exposed

Table 2 Predominant differentially expressed proteins after lethal exposure to copper surfaces

Accession #	Gene	Description	Functional category	Fold change ^a
<i>Up-regulated after copper exposure</i>				
NP_417350	YqeA	Predicted amino acid kinase	E	187.9
NP_418145	CbrA	Predicted oxidoreductase with FAD/NAD(P)-binding domain	C	53.8
NP_415003	DnaX	DNA polymerase III/DNA elongation factor III	L	22.7
NP_417877	MalT	DNA-binding transcriptional activator/maltotriose-ATP-binding protein	K	12.3
NP_418007	BisC	Biotin sulfoxide reductase	C	12.3
NP_417930	YhhS	Predicted transporter	G	10.4
NP_415810	SapA	Predicted antimicrobial peptide transporter subunit	E	9.4
NP_416841	YfcV	Predicted fimbrial-like adhesin protein	None	9.4
NP_415612	AcpP	Acyl carrier protein	I	8.7
NP_416543	RfbA	Glucose-1-phosphate thymidyl transferase	M	8.5
NP_415567	OpgH	Glycosyl transferase	M	8.5
NP_415193	YbeZ	Predicted protein with nucleoside triphosphate hydrolase domain	T	8.5
NP_414941	YajC	SecYEG protein translocase auxillary subunit	N	8.4
NP_415477	OmpA	Outer membrane protein A	M	6.1
NP_415647	PhoQ	Sensory histidine kinase in two-component regulatory system with PhoP	T	5.7
NP_418246	HemY	Predicted protoheme IX synthesis protein	H	4.7
NP_414626	FtsI	Transpeptidase involved in septal peptidoglycan synthesis	M	4.7
NP_416188	YdhV	Predicted oxidoreductase	C	4.7
NP_417134	YpjA	Adhesin-like autotransporter	Q	4.4
NP_414970	Tig	Peptidyl-prolyl cis/trans isomerase (trigger factor)	O	3.0
<i>Down-regulated after copper exposure</i>				
NP_418228	TrxA	Thioredoxin 1	O	3.2
NP_415840	Tpx	Lipid hydroperoxide peroxidase	O	3.2
NP_418376	MetF	5,10-methylenetetrahydrofolate reductase	E	4.9
NP_418567	GroL	Cpn60 chaperonin GroEL, large subunit of GroESL	O	12
NP_418411	RplA	50S ribosomal subunit protein L1	J	12
NP_418366	HslU	Molecular chaperone and ATPase component of HslUV protease	O	12
NP_415257	SucD	Succinyl-CoA synthetase, NAD(P)-binding, alpha subunit	C	12
NP_415138	AhpC	Alkyl hydroperoxide reductase	O	13
NP_417211	FhlA	DNA-binding transcriptional activator	K	13
NP_418104	LigB	DNA ligase, NAD(+)-dependent	L	14
NP_417172	LuxS	S-ribosylhomocysteinase	T	16
NP_417798	TufA	Protein chain elongation factor EF-Tu	J	17
NP_417703	Mdh	Malate dehydrogenase, NAD(P)-binding	C	19
NP_417768	RplX	50S ribosomal subunit protein L24	J	19
NP_417799	FusA	Protein chain elongation factor EF-G, GTP-binding	J	20
NP_418621	RpsF	30S ribosomal subunit protein S6	J	22
NP_415006	HtpG	Molecular chaperone HSP90 family	O	25
NP_415268	TolB	Periplasmic protein	N	33
NP_417083	ClpB	Protein disaggregation chaperone	O	36
NP_418438	AceB	Malate synthase A	C	65

^a Compared to control cells

sub-lethally to copper. Most prominently a sensory histidine kinase in two-component regulatory system with PhoP (b1129, PhoQ) involved in adaptation to low Mg^{2+} environments, a cytochrome d terminal oxidase (b0733, *CydA*) and a putative major facilitator superfamily transporter (b3473, *YhhS*). It is also interesting that 44 proteins (70%) of the up-regulated proteome after lethal exposure were not detected in the control proteome dataset.

In contrast, the down-regulated sub-proteome was dominated by proteins grouped with a role in translation (14 proteins) with six of them showing more than 10-fold decrease in abundance, posttranslational modification (10 proteins), energy production and conversion (10 proteins) and carbohydrate transport and metabolism (8 proteins). Other than those involved in translation, the proteins that showed drastic changes in their expression levels include the *Cpn60* chaperonin *GroL* (b4143, 12-fold), heat shock protein *ClpB* (b2592, 36-fold), a molecular chaperone of the HSP90 family (b0473, *HtpG*, 25-fold) and malate synthase A (b4014, *AceB*, 65-fold). Besides *AceB*, other TCA cycle enzymes identified in this study were also down-regulated after exposure to copper, especially isocitrate lyase (b4015, *AceA*), succinyl-CoA synthetase (b0729, *SucD*), succinate dehydrogenase, (b0723, *SdhA*) and malate dehydrogenase (b3236, *Mdh*).

Out of the 50 proteins that were up-regulated after sub-lethal exposure to copper, 14 proteins (28%) showed significantly lower expression levels as the exposure time increased to lethal levels. The protein that showed the maximum down-regulation (14-fold) was thiamine binding transketolase (b2935, *TktA*) of the pentose-phosphate pathway, whereas *TktA* was significantly up-regulated (14-fold) after sub-lethal stress. Other proteins showing similar trends in abundance, were acyl coenzyme A dehydrogenase (b0221, *FadE*), protoheme IX synthesis protein (b3802, *HemY*), acid-resistance protein (b3509, *HdeB*), outer membrane protein A (b0957, *OmpA*), and a cold shock stress response protein (b1823, *CspC*).

Stainless steel-responsive proteome

Stainless steel coupons were used as another metal surface to measure the differential protein expression in comparison to elemental copper. This provided the

opportunity to elucidate changes solely caused by desiccation stress upon exposure to dry metal surfaces. The proteomic profiles obtained for the unexposed *E. coli* cells and cells exposed to stainless steel and copper surfaces for the same time-periods (sub-lethal and lethal) were compared. While the size of the differentially expressed proteome remained in similar ranges (21–27% of the total proteome) when the cells were exposed either to stainless steel or copper surfaces sub-lethally or lethally, the proportion of the up- or down-regulated sub-proteome differed significantly. Sub-lethal exposure to copper surfaces resulted in 55% of the differential proteome being down-regulated compared to 27% on steel surfaces. When the exposure time was increased to 5 min, a greater percentage of proteins (63%) remained up-regulated on steel surfaces; however, the majority of the differentially expressed proteins were down-regulated (54%) on copper surfaces (Fig. 2).

Differential protein expression on stainless steel and copper surfaces

A comparative analysis of proteomic profiles of *E. coli* cells exposed sub-lethally to steel and copper surfaces revealed 22 proteins with a statistically significant differential abundance between the surfaces (Table S4, supplemental data). The sub-proteome that was up-regulated on copper surfaces compared to stainless steel comprised 13 proteins with 5 proteins exclusively identified in copper

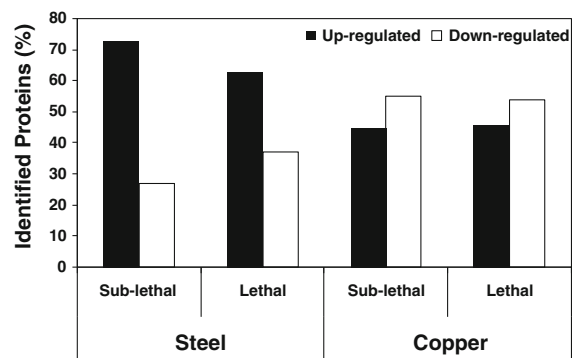


Fig. 2 Differential expression of proteins identified from cells after exposure to stainless steel versus copper surfaces. Number of identified proteins up- or down-regulated is shown as percentages of the differentially expressed proteins in each category. Please note, the terms sub-lethal and lethal refer to the time needed for killing on copper surfaces

exposed cells such as the quorum signaling molecule, autoinducer-2 (AI-2) kinase (IsrK).

Among the proteins that were more abundant on copper surfaces compared to stainless steel, the Mal regulon activator (b3418, MalT), the porin OmpA (b0957), cell division inhibitor MinD (b1175), alkyl hydroperoxide reductase (b0605, AhpC) and RNA helicases (b3780, RhlB and b0797, RhlE) were prominent. Among the nine proteins that were down-regulated following copper exposure, few proteins showed drastic reduction in their expression levels including the adhesin-like autotransporter (b2647, YpjA), 5,10-methylenetetrahydrofolate reductase (b3941, MetF), protein chain elongation factor EF-G (b3340, FusA), stress response protein HdeA (b3510) and Cpn60 chaperonin GroL (b4143). Hyper-osmotically inducible periplasmic protein (b4376, OsmY) and two acid-resistance proteins (b3509, HdeB and b3510, HdeA) were also up-regulated on steel surfaces. HdeAB respond to low pH and also exhibit chaperone activity below pH 3 by suppressing the aggregation of denatured periplasmic proteins non-specifically.

Twenty-four proteins exhibited significant differential abundance between stainless steel and the copper surfaces after lethal exposure (Table S4, supplemental data). The up-regulated sub-proteome comprised 21 proteins, e.g., Cpn10 chaperonin GroS (b4142), outer membrane protein A (b0957, OmpA), glutathione synthetase (b2947, GshB) and a stress responsive protease (b3153, YhbO). The down-regulated sub-proteome at lethal exposure was a subset of those already down-regulated after sub-lethal exposure with acid-resistance proteins being prominent (6–16-fold changes). Low-abundant, copper-response proteins such as a sensory histidine kinase CusS (b0570) and copper/silver efflux system protein CusB (b0574) were also identified in copper exposed cells though the differential levels were not significant. Additional copper response proteins (with two unique peptides of 80% peptide probability) were also detected in these datasets including the copper/silver efflux pump CusA (b0575) and its associated outer membrane factor CusC (b0572), the multicopper oxidase CueO (b0123), copper-requiring tyramine oxidase TynA (b1386) and CueR (b0487) the transcriptional regulator of CueO and CopA. The latter two were exclusively detected in the copper-responsive proteome.

Post translational modifications of proteins

To obtain more in-depth proteome information, the datasets acquired for the control and copper-exposed cells, were analyzed for differentially expressed, modified proteins with a particular emphasis on post translational modifications related to redox stress.

Several proteins identified with post translational modification of lysine, arginine, proline and threonine side chains were differentially abundant after exposure to copper at either of the two time points tested. Forty proteins were detected having peptides with modifications of lysine residue to amino adipic semialdehyde; 30 proteins with arginine to glutamic semialdehyde; 24 proteins with proline to 2-pyrrolidone and 13 proteins with threonine to 2-amino-3-ketobutyric acid (Table S5, supplemental data). Post translational modification analysis for peptides with oxidation of methionine and cysteine residues and oxidation of aromatic amino acids (phenylalanine, histidine, tyrosine and tryptophan) was also done on both exposed and unexposed proteome datasets. Among them, oxidation of methionine residues was predominant with 24 proteins showing significant differential abundance following copper exposure. Proteins with posttranslational modifications of histidine (six proteins), phenylalanine (ten proteins), cysteine (one protein), tryptophan (five proteins) and tyrosine (one protein) were also differentially expressed. Among the 135 proteins with different posttranslational modifications, we found eleven proteins that were modified by at least two of the tested different modifications and four with more than two. These included chaperonin GroL (b4143), a periplasmic protein (b4376, OsmY), and pyruvate dehydrogenase (b0115, AceF). In several proteins, peptides with modified residues were identified at increased levels, often by more than 10-fold, after exposure to copper. Predominant proteins include an outer membrane pore protein (b1377, OmpN, 100-fold, lysines), a predicted protein of unknown function (b0329, YahO, 29-fold, methionines), thio-sulfate transporter protein (b2425, CysP, 25-fold, lysines), membrane bound hydrogenase 2 (b2997, HybO, 23-fold, histidines), predicted carbohydrate kinase (b4167, YjeF, 20-fold, threonines) and chaperone Hsp70 (b0014, DnaK, 11-fold, methionines). Proteins belonging to the functional category of transport and metabolism of carbohydrates (G) and

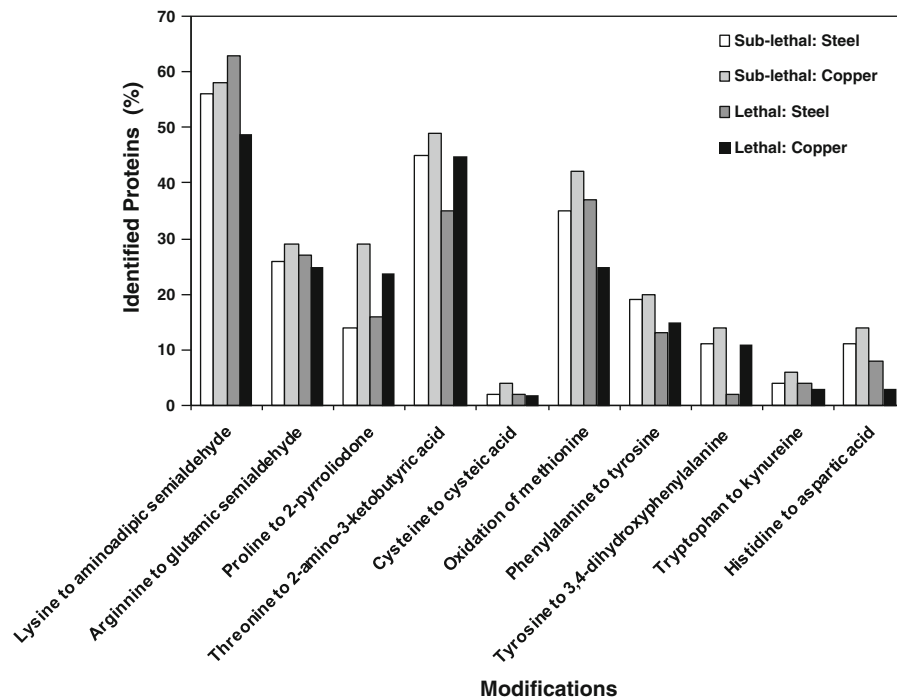


Fig. 3 Oxidatively modified proteins identified from cells exposed to steel or copper surfaces. The number of proteins with oxidative posttranslational modifications were expressed

amino acids (E), energy production and conversion (C) and translation, ribosomal structure and biogenesis (J) were extensively modified. In general, modified proteins were distributed in a variety of functional classes suggesting that posttranslational modifications were extensively involved in all the cellular processes that are active when cells had been exposed to copper.

To explore further the role of posttranslational modifications in *E. coli* cell responses to different metal surfaces, the proteome datasets of cells exposed sub-lethally and lethally to copper and steel surfaces were analyzed for above mentioned posttranslational modifications and compared (Fig. 3). At both time points tested, more proteins with modified proline residues were identified when the cells were exposed to copper (24–29% of total identified proteins) compared to steel (14–16%). A similar trend was also shown in the case of proteins with modification of threonine to 2-amino-3-ketobutyric acid. After sub-lethal exposure, more proteins with oxidized methionine residues were identified in the copper-responsive proteome in contrast to steel. Conversely

as the percentage of total proteins identified. Please note, the terms sub-lethal and lethal refer to the time needed for killing on copper surfaces

after lethal exposure, a higher number was detected in the steel-responsive proteome than in copper challenged cells. Together, these results indicate that modifications of proline and threonine might be specific for cells after contact to copper surfaces whereas other posttranslational modifications that were also identified after contact to stainless steel were likely responses to desiccation stress.

Discussion

Hospital trials in different countries have proven copper surfaces to be highly antimicrobial (Casey et al. 2010; Mikolay et al. 2010). In spite of the successful application of this new and promising tool for curbing hospital acquired infections we have only begun to understand the molecular mode-of-action of metallic copper (as recently reviewed in Grass et al. 2011). To the best of our knowledge no global, quantitative proteomic analysis of bacteria exposed to dry copper has yet been published. Our study of differential protein expression profiling allowed us to

analyze the response of *E. coli* to acute copper surface stress. This resulted in the identification of proteins that are involved in general stress response, cell envelope and capsule polysaccharide biogenesis, signal transduction, carbohydrate metabolism, energy production and DNA-replication and repair. The functional category of secondary metabolite biosynthesis, transport and catabolism was highly represented in the copper-responsive proteome with many efflux proteins and multidrug resistance proteins.

Identification and the high abundance of cell envelope and capsule polysaccharide biogenesis proteins in the copper-responsive proteome support the hypothesis, that the cell envelope of metallic copper-exposed cells suffered most of the damage (Espirito Santo et al. 2011). Though many of the proteins belong to the functional category of DNA-replication, recombination and repair were present, typical proteins involved in DNA-repair were not identified in the copper exposed cells. One of the up-regulated proteins identified in the copper proteome, DnaX, belongs to this functional category and is involved in DNA replication by DNA polymerase III but not specifically in repair (Hubscher and Kornberg 1979). Thus, our data does not support an alternative theory on the mode-of-action, in which DNA is the primary target of exposure to metallic copper (Warnes et al. 2010). Previously, in chronically copper ion-exposed growing *E. coli* cells also no increase in oxidative DNA damage in response to the stress was observed (Macomber et al. 2007). Similarly, our recent studies on the killing mechanisms mediated by metallic copper of bacteria (Espirito Santo et al. 2011) and yeasts (Quaranta et al. 2011) did not find an increase in mutation rates or DNA-damage in cells exposed for a sub-lethal time period.

Up-regulation of the iron storage and detoxification protein, bacterioferritin (b3336, Bfr), is likely related to the chelation of excess cytosolic iron cations as a consequence of iron-sulfur cluster damage mediated by copper ions. This kind of damage was recently identified as major contributor of copper-ion toxicity in *E. coli* (Macomber and Imlay 2009) and *Bacillus subtilis* (Chillappagari et al. 2010). Thus, in this case copper-toxicity is indirect by copper-mediated release of the Fenton-agent ferrous iron. Bacterioferritin might counter this stress through iron-oxidation and storage in its hollow multimeric shell. Along that line we investigated

oxidative changes in proteins mediated by copper ion stress. Mining of the proteome datasets for posttranslational modifications revealed that proteins with oxidative modifications of lysine to amino adipic semialdehyde and arginine to glutamic semialdehyde were highly prevalent and differentially expressed in copper-responsive proteomes. Glutamic and amino adipic acid semialdehydes are reported as the main carbonyl products of the metal catalyzed oxidation of proteins (Requena et al. 2001). However, it has been shown that aging-, starvation-, and stress-induced carbonylation does not affect the proteome uniformly (Levine and Stadtman 2001) and in *E. coli*, the targeted proteins include the Hsp70 and Hsp60 chaperones, elongation factors, EF-Tu and EF-G, glutamine synthetase, glutamate synthase, aconitase, malate dehydrogenase, and pyruvate kinase (Dukan and Nystrom 1998; Dukan and Nystrom 1999; Tamarit et al. 1998). In our proteome datasets, carbonylation modifications of lysine and arginine residues were identified in many proteins especially pyruvate kinase, malate dehydrogenase, elongation factor EF-Tu, and in Hsp70 and Hsp60 chaperones. In addition, proteins of copper-stressed cells exhibited higher levels of proline to 2-pyrrolidone and threonine to 2-amino-3-ketobutyric acid modifications compared to stainless steel. At this time, however, our data does not support conclusions on the significance and relevance of these modifications. Yet, it is noteworthy that contact to both copper and stainless steel elicited extensive amino acid residue oxidations, most of them likely caused by desiccation stress of cells on these dry metallic surfaces.

Transcriptomic studies on response of *E. coli* to copper ion stress in growing cultures identified the genes of the copper ion homeostasis systems, Cus, CueO or CopA, among those being prominently up-regulated (e.g., Kershaw et al. 2005). The multi-copper oxidase, CueO (b0123), is involved in periplasmic detoxification of copper by oxidizing Cu(I) to Cu(II) and thus converting copper into a less toxic species. CueO binds 4–5 copper ions per monomer and plays a significant role in copper homeostasis under aerobic conditions (Roberts et al. 2002). However, its differential expression between exposed and unexposed cells was not statistically significant and failed to meet the 2-fold cut off. Five more proteins with a function directly related to the presence of copper ions in the cell environment were

also identified including the copper/silver efflux system proteins (b0575, CusA; b0574, CusB and b0572, CusC), a copper homeostasis protein (b1874, CutC) and a lipoprotein involved in copper homeostasis and adhesion (b0192, CutF). These proteins also failed to make the cut-off because they were assigned with only one unique peptide.

Other proteins that were up-regulated after contact to copper had broader stress-related functions. Similar to our findings, general stress proteins, DnaK, superoxide dismutase, OsmC and cold shock proteins were also more abundant in chronically copper stressed cells of the Gram-positive *Kineococcus radiotolerans* (Bagwell et al. 2010). This further emphasizes the broad and unspecific extend of stress mediated by copper both in acute and chronic exposure which tend to obscure the specific targets of copper toxicity such as exposed iron-sulfur clusters in enzymes (Macomber and Imlay 2009).

Surprisingly, proteins implicated in stress response, such as alkyl hydroperoxide reductase AphC (b0605), lipid hydroperoxide peroxidase Tpx (b1324) and thioredoxin I TrxA (b3781) were down-regulated in copper challenged cells compared to control cells. The expression levels of a variety of chaperones (but not DnaK) were also decreased after cells were exposed to copper. This might be a general consequence of down-regulated protein synthesis. In the highly metal-resistant bacterium *Cupriavidus metallidurans* CH34 general stress response chaperones such as GroEL and GroES were also not up-regulated in copper ion-challenged cultures (Monchy et al. 2006). Many TCA cycle enzymes identified in our study were also significantly down-regulated after exposure to copper. Similar observations were made in *E. coli* cells exposed to cadmium in which many energy conversion enzymes including TCA cycle enzymes either disappeared or were down-regulated (Isarankura-Na-Ayudhya et al. 2009).

The sulfur-containing amino acid homocysteine (Hcy) is known to be toxic and is implicated in a number of life-threatening human diseases (Fraser et al. 2006). Copper is known to catalyze hydrogen peroxide generation from Hcy in endothelial cells (Starkebaum and Harlan 1986) but currently there is no evidence that Hcy induces oxidative stress in *E. coli*. In *E. coli* an undefined amount of Hcy is generated by the action of LuxS on S-ribosylhomocysteine. The significant down-regulation of LuxS

(b2687, 15-fold) could be a strategy to reduce the level of Hcy in the presence of excess of copper to minimize the oxidative stress. In *E. coli*, Hcy is converted to methionine in a methylation reaction, which requires a methyl donor, 5-methyltetrahydrofolate. The synthesis of 5-methyltetrahydrofolate requires methylenetetrahydrofolate reductase (b3941, MetF) (Fraser et al. 2006). The lower levels of MetF (5-fold) after 5 min of exposure could stem from the low amount of Hcy entering the methionine biosynthetic pathway. Hcy is also known to trigger a decrease in glutathione peroxidase in human endothelial cells and also acts to block the H₂O₂-mediated activation of Hsp70 expression (Outinen et al. 1998). In the present study, glutathione peroxidase (b1710, BtuE) which was abundant in the control cells, was not identified in the copper exposed proteome. It is noteworthy that the expression of glutathione synthetase (b2947, GshB) was significantly enhanced (5-fold) in the copper exposed cells compared to the stainless steel. Previously, it was shown that the tripeptide glutathione plays an important role as intracellular copper ion buffer in copper tolerance in *E. coli* (Helbig et al. 2008) and this might have also lead to an increase of glutathione as a response to contact to copper surfaces. However, glutathione biosynthesis was not increased in copper ion treated *K. radiotolerans* (Bagwell et al. 2010).

Overall, we identified a variety of proteins differently abundant in copper surface-exposed cells than in unchallenged or stainless steel-exposed control cells. Since this is the first quantitative proteomic analysis of cells acutely stressed by dry metallic copper surfaces, comparison of our findings to other studies concerning the cellular responses to chronic copper ion exposure is difficult. However, results outlined in the present study provided the first glimpse into the predominant proteins and functional categories involved in the cell response mechanisms towards metallic copper stress and will serve as a basis for future proteomic studies to elucidate the acute copper ion stress responses.

Acknowledgments The mass spectrometry analysis was conducted at the Proteomic and Metabolomic Core facility, Redox biology center, UNL and the research was supported by NIH Grant Number P20 RR-17675, from the National Center for Research Resources. The contents of this article are solely the responsibility of the authors and do not represent the official views of the NIH. We also acknowledge funds from the International Copper Association (ICA), the Copper

Development Association (CDA), and a University of Nebraska-Lincoln Office of Research Faculty Seed grant to G.G. C.E.S. was supported by a *Fundação para a Ciência e Tecnologia*, Portugal, graduate fellowship.

References

- Bagwell CE, Hixson KK, Milliken CE, Lopez-Ferrer D, Weitz KK (2010) Proteomic and physiological responses of *Klebsiella aerogenes* to copper. *PLoS One* 5:e12427
- Casey AL, Adams D, Karpanen TJ, Lambert PA, Cookson BD, Nightingale P, Miruszenko L, Shillam R, Christian P, Elliott TSJ (2010) Role of copper in reducing hospital environment contamination. *J Hosp Infect* 74:72–77
- Chillappagari S, Seubert A, Trip H, Kuipers OP, Marahiel MA, Miethke M (2010) Copper stress affects iron homeostasis by destabilizing iron-sulfur cluster formation in *Bacillus subtilis*. *J Bacteriol* 192:2512–2524
- Dukan S, Nystrom T (1998) Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. *Genes Dev* 12:3431–3441
- Dukan S, Nystrom T (1999) Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells. *J Biol Chem* 274:26027–26032
- Elguindi J, Wagner J, Rensing C (2009) Genes involved in copper resistance influence survival of *Pseudomonas aeruginosa* on copper surfaces. *J Appl Microbiol* 106:1448–1455
- Elguindi J, Moffitt S, Hasman H, Andrade C, Raghavan S, Rensing C (2011) Metallic copper corrosion rates, moisture content, and growth medium influence survival of copper ion-resistant bacteria. *Appl Microbiol Biotechnol*. doi:10.1007/s00253-010-2980-x
- Espirito Santo C, Taudte N, Nies DH, Grass G (2008) Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. *Appl Environ Microbiol* 74:977–986
- Espirito Santo C, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Chang CJ, Grass G (2011) Bacterial killing by dry metallic copper surfaces. *Appl Environ Microbiol* 77:794–802
- Fraser KR, Tuite NL, Bhagwat A, O'Byrne CP (2006) Global effects of homocysteine on transcription in *Escherichia coli*: induction of the gene for the major cold-shock protein, CspA. *Microbiology* 152:2221–2231
- Gardy JL, Laird MR, Chen F, Rey S, Walsh CJ, Ester M, Brinkman FS (2005) PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. *Bioinformatics* 21:617–623
- Grass G, Rensing C, Solioz M (2011) Metallic copper as an antimicrobial surface. *Appl Environ Microbiol* 77:1541–1547
- Helbig K, Bleuel C, Krauss GJ, Nies DH (2008) Glutathione and transition-metal homeostasis in *Escherichia coli*. *J Bacteriol* 190:5431–5438
- Hubscher U, Kornberg A (1979) The delta subunit of *Escherichia coli* DNA polymerase III holoenzyme is the *dnaX* gene product. *Proc Natl Acad Sci USA* 76:6284–6288
- Isarankura-Na-Ayudhya P, Isarankura-Na-Ayudhya C, Treeratanapaiboon L, Kasikun K, K T, Prachayasittikul V (2009) Proteomic profiling of *Escherichia coli* in response to heavy metals stress. *Eur J Sci Res* 25:679–688
- Kao WC, Chen YR, Yi EC, Lee H, Tian Q, Wu KM, Tsai SF, Yu SS, Chen YJ, Aebersold R, Chan SI (2004) Quantitative proteomic analysis of metabolic regulation by copper ions in *Methylococcus capsulatus* (Bath). *J Biol Chem* 279:51554–51560
- Kershaw CJ, Brown NL, Constantinidou C, Patel MD, Hobman JL (2005) The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. *Microbiology* 151:1187–1198
- Levine RL, Stadtman ER (2001) Oxidative modification of proteins during aging. *Exp Gerontol* 36:1495–1502
- Liu H, Sadygov RG, Yates JR III (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem* 76:4193–4201
- Macomber L, Imlay JA (2009) The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc Natl Acad Sci USA* 106:8344–8349
- Macomber L, Rensing C, Imlay JA (2007) Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. *J Bacteriol* 189:1616–1626
- Magnani D, Barre O, Gerber SD, Solioz M (2008) Characterization of the CopR regulon of *Lactococcus lactis* IL1403. *J Bacteriol* 190:536–545
- Mehtar S, Wiid I, Todorov SD (2007) The antimicrobial activity of copper and copper alloys against nosocomial pathogens and *Mycobacterium tuberculosis* isolated from healthcare facilities in the Western Cape: an in vitro study. *J Hosp Infect* 68:45–51
- Mikolay A, Huggett S, Tikana L, Grass G, Braun J, Nies DH (2010) Survival of bacteria on metallic copper surfaces in a hospital trial. *Appl Microbiol Biotechnol* 87:1875–1879
- Miller CD, Pettee B, Zhang C, Pabst M, McLean JE, Anderson AJ (2009) Copper and cadmium: responses in *Pseudomonas putida* KT2440. *Lett Appl Microbiol* 49:775–783
- Molteni C, Abicht HK, Solioz M (2010) Killing of bacteria by copper surfaces involves dissolved copper. *Appl Environ Microbiol* 76:4099–4101
- Monchy S, Benotmane MA, Wattiez R, van Aelst S, Auquier V, Borremans B, Mergeay M, Taghavi S, van der Lelie D, Vallaeyts T (2006) Transcriptomic and proteomic analyses of the pMOL30-encoded copper resistance in *Cupriavidus metallidurans* strain CH34. *Microbiology* 152:1765–1776
- Nägele E, Vollmer M, Horth P (2003) Two-dimensional nano-liquid chromatography-mass spectrometry system for applications in proteomics. *J Chromatogr A* 1009:197–205
- Nandakumar R, Madayiputhiya N, Fouad AF (2009) Proteomic analysis of endodontic infections by liquid chromatography-tandem mass spectrometry. *Oral Microbiol Immunol* 24:347–352
- Noyce JO, Michels H, Keevil CW (2006) Potential use of copper surfaces to reduce survival of epidemic methicillin-resistant *Staphylococcus aureus* in the healthcare environment. *J Hosp Infect* 63:289–297

- Outinen PA, Sood SK, Liaw PC, Sarge KD, Maeda N, Hirsh J, Ribau J, Podor TJ, Weitz JI, Austin RC (1998) Characterization of the stress-inducing effects of homocysteine. *Biochem J* 332(Pt 1):213–221
- Quaranta D, Krans T, Espirito Santo C, Elowsky CG, Domaille DW, Chang CJ, Grass G (2011) Mechanisms of yeast contact-killing on dry metallic copper surfaces. *Appl Environ Microbiol* 77:416–426
- Requena JR, Chao CC, Levine RL, Stadtman ER (2001) Glutamic and amino adipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* 98:69–74
- Roberts SA, Weichsel A, Grass G, Thakali K, Hazzard JT, Tollin G, Rensing C, Montfort WR (2002) Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. *Proc Natl Acad Sci USA* 99:2766–2771
- Sharma S, Sundaram CS, Luthra PM, Singh Y, Sirdeshmukh R, Gade WN (2006) Role of proteins in resistance mechanism of *Pseudomonas fluorescens* against heavy metal induced stress with proteomics approach. *J Biotechnol* 126:374–382
- Starkebaum G, Harlan JM (1986) Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J Clin Invest* 77:1370–1376
- Tamarit J, Cabiscol E, Ros J (1998) Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J Biol Chem* 273:3027–3032
- Tatusov RL, Galperin MY, Natale DA, Koonin EV (2000) The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res* 28:33–36
- Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M, Parsek MR (2006) Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J Bacteriol* 188:7242–7256
- Warnes SL, Green SM, Michels HT, Keevil CW (2010) Bicidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNAs. *Appl Environ Microbiol* 76:5390–5401
- Weaver L, Michels HT, Keevil CW (2010) Potential for preventing spread of fungi in air-conditioning systems constructed using copper instead of aluminium. *Lett Appl Microbiol* 50:18–23
- Yamamoto K, Hirao K, Oshima T, Aiba H, Utsumi R, Ishihama A (2005) Functional characterization in vitro of all two-component signal transduction systems from *Escherichia coli*. *J Biol Chem* 280:1448–1456