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Proteomic Adaptations to Starvation Prepare *Escherichia coli* for Disinfection Tolerance

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Abstract

Despite the low nutrient level and constant presence of secondary disinfectants, bacterial re-growth still occurs in drinking water distribution systems. The molecular mechanisms that starved bacteria use to survive low-level chlorine-based disinfectants are not well understood. The objective of this study is to investigate these molecular mechanisms at the protein level that prepare starved cells for disinfection tolerance. Two commonly used secondary disinfectants chlorine and monochloramine, both at 1 mg/L, were used in this study. The proteomes of normal and starved *Escherichia coli* (K12 MG1655) cells were studied using quantitative proteomics. Over 60-min disinfection, starved cells showed significantly higher disinfection tolerance than normal cells based on the inactivation curves for both chlorine and monochloramine. Proteomic analyses suggest that starvation may prepare cells for the oxidative stress that chlorine-based disinfection will cause by affecting glutathione metabolism. In addition, proteins involved in stress regulation and stress responses were among the ones up-regulated under both starvation and chlorine/monochloramine disinfection. By comparing the fold changes under different conditions, it is suggested that starvation prepares *E. coli* for disinfection tolerance by increasing the expression of enzymes that can help cells survive chlorine/monochloramine disinfection. Protein co-expression analyses show that proteins in glycolysis and pentose phosphate pathway that were up-regulated under starvation are also involved in disinfection tolerance. Finally, the production and detoxification of methylglyoxal may be involved in the chlorine-based disinfection and cell defense mechanisms.

Keywords

Disinfection tolerance; starvation; quantitative proteomics; chlorine; monochloramine

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1. INTRODUCTION

Microbial re-growth in drinking water distribution systems (DWDSs) poses a threat to drinking water safety (Falkinham et al. 2001, LeChevallier et al. 1996). Although primary disinfection is carried out at the end of the drinking water treatment train and secondary disinfection is accomplished by adding low levels of disinfectants into DWDSs, microbial re-growth is frequently detected in biofilms and bulk water of DWDSs (Berry et al. 2006). Assimilable organic carbon, the portion of total organic carbon that can be readily utilized by microbes for growth, ideally should not exceed 50 µg/L in DWDSs to avoid microbial growth (Lechevallier et al. 1991). Given the low disinfectant and low nutrient levels in DWDSs, understanding how bacteria metabolize under mild disinfection and starvation stresses is important to develop methods to control microbial re-growth in DWDSs.

Free chlorine and monochloramine are commonly used secondary disinfectants in the US. Although their disinfection effectiveness has been recognized, their mechanisms of inactivating bacteria need further investigation (Hwang et al. 2012, Wang et al. 2014). Free chlorine is often considered a nonselective oxidant to react with cellular components and affect metabolic processes (Albrich and Hurst 1982). For example, it causes irreversible aggregation of thermolabile proteins (Winter et al. 2008), damage DNA (Dukan and Touati 1996a) and disrupts DNA synthesis (Mckenna and Davies 1988), and interrupts the electron transport chain (Hurst et al. 1991). Monochloramine is being increasingly used as a secondary disinfectant due to its lower potency to form disinfection byproducts than free chlorine, however, little is known about its mode of action. Some studies report that monochloramine can oxidize sulfhydryl groups in amino acids (Jacangelo et al. 1987), and the oxidation is sometimes reversible (Watters et al. 1989).

Efforts have been made to elucidate the mechanisms bacteria use to survive disinfection. Earlier studies focused on individual mechanisms in disinfection tolerance, such as synthesis of glutathione as an oxidant scavenger and activator of cell defense systems (Saby et al. 1999), and *rpoS*- and *oxyR*-governed adaptive responses (Dukan and Touati 1996a). Some recent studies used microarray to investigate the comprehensive cellular responses to chlorine-based disinfection. One study shows that upon exposure to 390 mg/L free chlorine, several classes of genes in *E. coli* were significantly up-regulated, such as genes responsive to oxidative stresses, genes encoding putative oxidoreductases, and genes related to cysteine biosynthesis and iron-sulfur cluster systems (Wang et al. 2009). Similarly, upon exposure to 1 mg/L monochloramine, *E. coli* up-regulated genes related to redox responses, oxidoreductase synthesis, and cell envelope integrity response (Berry et al. 2010), and the cellular responses were complex and dynamic (Holder et al. 2013). Given that mRNA transcripts do not always directly correlate with the expression levels of the encoded proteins due to posttranslational modification (Cox and Mann 2011), there is a need to investigate the cellular response to disinfection at the protein level.

Several studies report that starved cells exhibited higher disinfection tolerance than normal cells (Cherchi and Gu 2011, Stewart and Olson 1992). However, the molecular mechanism under the phenomenon has never been elucidated. Bacteria undergo metabolic changes under starvation. For example, proteins related to general stress responses, such as the sigma

factor (RpoS)(Martínez-Gómez et al. 2012), and oxidative stress responses are up-regulated, a phenomenon called multiresistance response or cross-protection (Rangel 2011). The cross-protection phenomenon has also been observed in starved *E. coli* cells that up-regulated proteins responsive to stress conditions such as heat (Jenkins et al. 1988), oxidative stress (Jenkins et al. 1988), and osmotic stress (Jenkins et al. 1990). There is a knowledge gap in understanding the molecular response governing the cross-protection between starvation and chlorine-based disinfection.

The objective of this study is to investigate the molecular mechanisms at the protein level that prepares starved cells for elevated disinfection tolerance. *E. coli* was used as model bacterial species in this study, and both chlorine and monochloramine were included. A shotgun quantitative proteomic approach was employed for proteomic analyses. It is expected that the outcome from this study can facilitate the development of approaches to limit and control microbial re-growth in DWDSs.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Growth Conditions

Escherichia coli K12 AT980 cultures were grown in Luria-Bertani (LB) medium at 250 rpm at 37°C. One set of cultures were harvested at the late exponential phase and were defined as *normal* cultures in this work. Another set of cultures were collected at the same time and then centrifuged and re-suspended in phosphate buffered saline (PBS, pH=8.0). These cultures were then starved for 24 hours at 20°C before harvesting (Saby et al. 1999, Tong et al. 2011) and were defined as *starved* cultures in this work. The 24-hr starvation period didn't cause significant changes in viable cell numbers ($p=0.40$).

2.2 Disinfection Experiments

The disinfection experiments were conducted at three different times. In each of the triplicate disinfection experiments, four 250-mL Erlenmeyer flasks covered by aluminum foil were used as batch reactors, two for chlorine and two for monochloramine. For each disinfectant, one flask received normal cells, while the other one received starved cells. Before disinfectants were added to the flasks, cells were washed with PBS (pH=8.0) twice and re-suspended in 100 mL PBS with a final cell density of $\sim 10^8$ CFU/mL. The chlorine stock solution was prepared by adding 10 μ L 11~15% sodium hypochlorite solution (Sigma-Aldrich, St. Louis, MO) into 100 mL Nanopure water. The monochloramine stock solution was prepared by adding 10 μ L 11~15% sodium hypochlorite solution and 2 g ammonium chloride into 100 mL Nanopure water (Larson and Marinas 2003). Both disinfectants were prepared immediately before inactivation experiments and their concentrations were determined using the DPD titrimetric method (Eaton et al. 2005). Chlorine or monochloramine was added to the batch reactors with a final concentration of 1 mg/L as Cl₂. The inactivation reaction was conducted in a 20°C water bath. At 0, 2, 5, 10, 20, 30, 40 and 60 min, 1 mL of the reaction solution was transferred to a 9-mL solution made with 5 mL of 0.01M PBS (pH=7.2) and 4 mL of 0.12% sodium thiosulfate pentahydrate to stop the inactivation reaction, and the remaining viable cells were enumerated LB agar plates.

Additionally, liquid samples were collected at 1, 10 and 60 min after the start of disinfection experiments to monitor disinfectant concentrations.

2.3 Inactivation Model and Statistical Analysis

The Chick-Watson inactivation model was used to simulate the inactivation profile of monochloramine (Berry et al. 2008)

$$\ln\left(\frac{N}{N_0}\right) = \begin{cases} 1 & \text{if } C^n t < C^n t_{\text{SHOULDER}} \\ -k(C^n t - C^n t_{\text{LAG}}) & \text{if } C^n t > C^n t_{\text{SHOULDER}} \end{cases}$$

N and N_0 are cell counts at time t and time zero, k is the inactivation rate constant, t_{SHOULDER} is the shoulder phase, n is an empirical parameter that describes the relative importance of the concentration of disinfectant which was set as 1 in this study, C is the disinfectant concentration, and t is inactivation time. T-test was used to determine if the k values between normal and starved cells was significantly different ($p < 0.05$) in the monochloramine experiments. The inactivation profile of *E. coli* with chlorine was not simulated, due to the lack of proper models that can fit the experimental data (Virto et al. 2005, Zhang et al. 2007).

2.4 Protein Extraction

A fourth disinfection experiment was conducted to collect biomass samples for proteomic analyses. A total of 16 flasks were included in the fourth disinfection experiment to cover the following experimental conditions: 2 disinfectant types (chlorine and monochloramine), 2 cell types (normal and starved cells), 2 time points (i.e., 0 and 10 min), and 2 replicates for each treatment combination. At each time point, 10 mL 0.12% sodium thiosulfate pentahydrate was added into each 90 mL reaction solution to terminate the inactivation reaction. The cells were harvested from 100ml of bacteria solution and then proteins were extracted from the cell pellet.

Proteins were extracted from the harvested cells as detailed in our previous work (Nandakumar et al. 2011). Specifically, after two washes using PBS (pH=8.0) cells were re-suspended in 1 mL of solution containing 50 mM ammonium bicarbonate, 8 M urea, and 1.5 mM phenylmethylsulfonyl fluoride (PMSF), and cells were lysed using bead-beating for 2.5 min. For every 0.5 min bead beating, the cells were moved on ice to chill for 5 min. Cell debris and glass beads were removed by centrifugation at 13,000×g for 10 min at 4°C. The proteins in supernatant were precipitated using acetone at -20°C overnight and were re-suspended in a solution containing 100 mM ammonium bicarbonate and 6 M urea. All 16 proteins samples were sent to the UNL Proteomics and Metabolomics Core Facility, where proteins were quantified using the BCA protein assay kit (Thermo Scientific, Waltham, MA). For each sample, 400 µg of proteins were reduced by 10 mM dithiothreitol, alkylated with 40mM iodoacetamide, and digested with sequencing-grade trypsin (Roche, Indianapolis, IN) at 1:50 trypsin to protein ratio at 37°C overnight. Tryptic peptides were desalted and concentrated using solid phase extraction (PepClean C-19 spin column,

Thermo Scientific, Rockford, IL), vacuum-dried, and stored at -80°C before 2D-LC MS/MS analyses.

2.5 Proteomic Analyses using 2D LC-MS/MS

Quantitative proteomics was performed on an Ultimate 3000 Dionex MDLC system (Dionex Corporation, CA) integrated with a nanospray source and LCQ Fleet Ion Trap mass spectrometer (Thermo Scientific, CA). The first dimensional separation was performed on a SCX column (Polysulfoethyl, 1mm I.D \times 15 cm, 5 μm , 300A, Dionex). 20 μL of samples were loaded onto first dimension SCX column and eluted using a salt gradient (0–600 mM). Selected fractions based on the UV absorbance of the eluted peptides were subjected to second dimension analysis. The second dimension separation incorporated an on-line sample pre-concentration and desalting using a monolithic C18 trap column (Pep Map, 300 μm I.D \times 5 mm, 100 \AA , 5 μm , Dionex). The sample was loaded on to the monolithic trap column at a flow rate of 40 $\mu\text{L}/\text{min}$. The desalted peptides were then eluted and separated on a C18 Pep Map column (75 μm I.D. \times 15 cm, 3 μm , 100 \AA , New Objective, USA) by applying an acetonitrile (ACN) gradient (ACN plus 0.1% formic acid, 90 minute gradient at a flow rate of 250 nL/min) and were 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 $^{\circ}\text{C}$; full scan m/z range, 400–2,000. Data acquisition was done in data dependent mode with 4 MS/MS spectra for every full scan, 5 microscans averaged for full scans and MS/MS scans, a 3 m/z isolation width for MS/MS isolations, and 35% collision energy for collision-induced dissociation.

2.6 Protein Identification and Quantitation

The MS/MS spectra were compared against the *E. coli* K12 proteome database using MASCOT (Version 2.2 Matrix Science, London, UK) with the following settings: enzyme-trypsin; missed cleavages-2; mass-monoisotopic; fixed modification-carbamidomethyl (C); peptide tolerance-1.5 Da; and MS/MS fragment ion tolerance-1 Da. Probability assessment of peptide assignments and protein identifications were performed using Scaffold 3.0 Proteome Software Inc., Portland, OR). The criteria for protein identification included the detection of at least two unique peptides per protein and a protein probability score of 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 proteins was developed using a label-free method of spectral counting with normalized spectral counts (Liu et al. 2004). Proteins expressing 2-fold change in abundance with $p < 0.05$ (Fisher's exact test on results from the duplicate protein extracts for each treatment combination) were considered as differentially expressed (Berry et al. 2010).

2.7 Protein Annotation and Interaction Analysis

Identified proteins were further analyzed using Blast2GO v2.6.5 for Gene Ontology (GO) annotation analysis. Proteins were also mapped into metabolic pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) (Moriya et al. 2007). The STRING database was used to predict the protein-protein interactions between co-expressed proteins (Szklarczyk et al. 2011).

3. RESULTS AND DISCUSSION

3.1 Inactivation of *E. coli* with Different Disinfectants

The inactivation kinetics of *E. coli* with different disinfectants (chlorine vs. monochloramine) and cell physiological conditions (normal vs. starved cells) are described using the inactivation curves in Figure 1, where CT is the product of disinfectant concentration (C) and disinfection time (T). The inactivation curves describe bacterial survival as a function of CT. With 1 mg/L chlorine, the shape of the inactivation profiles for normal and starved cells was similar. During the first 2 min, normal and starved *E. coli* cells decreased by about 1.3 and 1.1 orders of magnitude, respectively. After that, the inactivation rate decreased substantially and the cell number remained largely unchanged for normal and starved cells (Figure 1). Over the course of 60 minutes, there was a total of 1.5 log reductions in normal cells and a total of 1.3 log reductions in starved cells. Based on triplicate inactivation experiments, starved cells exhibited significantly higher survival ratio than normal cells at all time points tested (Figure 1, $p < 0.05$), suggesting starved cells had higher disinfection tolerance than normal cells.

For the disinfection experiments with 1 mg/L monochloramine, a 5-min shoulder phase occurred in normal cells and a 10-min shoulder phase occurred in the starved cells (Figure 1). Past the shoulder phase, the inactivation CT curve appeared to be linear for both cell types. The average inactivation rate constant k based on triplicate experiments was 0.24 and 0.14 (mg·min/L)⁻¹ for normal and starved cells, respectively, and the difference was statistically significant ($p < 0.05$), suggesting starved cells were more resistant to monochloramine than normal cells.

The short half-life of chlorine may be responsible for the shapes of the inactivation CT curves for chlorine disinfection. Chlorine concentration dropped to close to 0 mg/L 10 min after the start of the disinfection experiment, while the monochloramine concentration was still at 0.9 mg/L after 60 min (Table S1). Our findings are consistent with the literature (Zhang et al. 2007). Studies show that while 1 mg/L chlorine could quickly inactivate bacterial cells by oxidizing cell membrane components (Cho et al. 2010), most of the chlorine would be consumed in the first 5 min (Zhang et al. 2007), resulting in smaller overall inactivation efficiencies than monochloramine. Monochloramine is less oxidative than chlorine, so it was consumed less and lasted longer than chlorine.

3.2 Proteomic Response to Starvation

The proteomes of *E. coli* cells under each experimental condition were compared according to the scheme in Figure 2. Within each comparison, the fraction of the protein up-regulated accounted for less than 25% of the total proteins detected (Table S2). Comparison 1 identified a total of 48 proteins that were up-regulated in response to starvation. Five of them belonged to major metabolic pathways: malate synthase A (aceB, 2.1 fold) in the glyoxylate bypass, galactokinase (galK, 5.0 fold) in galactose metabolism, 2,3-bisphosphoglyceratedependent phosphoglycerate mutase (gpmA, 2.4 fold) in glycolysis, transketolase 2 (tktB, 3.5 fold) in pentose phosphate pathway, and thioredoxin/glutathione peroxidase (btuE, 2.8 fold) in glutathione metabolism (Figure 3).

Our results show that under starvation *E. coli* increased the synthesis of proteins that allowed the cells to utilize alternative substrates and respond to stresses. Malate synthase A (aceB) can be induced during cell growth on acetate, while galactokinase (galK) catalyzes the first step in the Leloir pathway of galactose metabolism. The up-regulation of these two proteins suggests that acetate and galactose, intermediate metabolites of several central metabolisms, may be used as alternative energy source by *E. coli* under starvation. Phosphoglycerate mutase (gpmA) can convert 2-phosphoglycerate to 3-phosphoglycerate in glycolysis. This enzyme was up-regulated in *Bacillus anthracis* that was exposed to hydrogen peroxide (Pohl et al. 2011), and appears to involve in the proteomic response of *E. coli* to chlorine-based disinfection (see below). Transketolase 2 (tktB) expression is often increased in stationary phase and positively regulated by the alternative sigma factor RpoS (Jung et al. 2005), which responds to different environmental stresses in a consistent way (Dong et al. 2011).

Another noticeable change was the up-regulation of the thioredoxin/glutathione peroxidase (btuE) in the glutathione metabolism. BtuE can be induced by oxidative stress and during stationary phase (Arenas et al. 2010), and can catalyze reactions in which antioxidants are converted to their oxidized forms in the presence of oxidants, for example, reduced glutathione (GSH) to glutathione disulfide (GSSG). One previous study reported that GSH played an important role in chlorine resistance (Saby et al. 1999). Together with the increase in GSH during starvation (Fahey et al. 1978), the increase in btuE expression reported in this study suggests that starvation may prepare cells for the oxidative stress that chlorine-based disinfection will cause by affecting glutathione metabolism.

3.3 Chlorine Disinfection on Normal and Starved Cells

Comparisons 2 and 3 in Figure 2 share the same reference proteome as Comparison 1, and reveal the proteomic response of normal and starved *E. coli* cells to chlorine disinfection, respectively. Proteins that were up-regulated in all three comparisons are listed in Table 1. The fold changes of these up-regulated proteins follow the general trend: disinfection of normal cells (Comparison 2) < starvation (Comparison 1) < disinfection of starved cells (Comparison 3). The trend shows that among the up-regulated proteins that responded to both starvation and chlorination, the fold change was higher in starved cells than in disinfected normal cells. Most importantly, further increase of expression in disinfected starved cells suggests that starvation may have prepared *E. coli* for disinfection tolerance by increasing the expression of enzymes that can help cells survive chlorine disinfection.

Stress regulators—Stress regulator hupB is the beta subunit of HU protein, which is an accessory factor stabilizing nucleoprotein complexes, and therefore, plays an important role in nucleotide organization (Ali Azam et al. 1999), regulation (Kar et al. 2005) and replication. HU protein is required for the expression of many genes in response to environmental changes and in adaptation to stress, including changes in osmolarity, acid stress, SOS induction, and anaerobiosis (Oberto et al. 2009).

Oxidative stress response proteins—Abundant in stationary phase *E. coli*, Dps can bind tightly to DNA in a non-sequence-specific manner and form a DNA-protein crystal to

protect DNA from damage. Dps is required to respond to starvation (Almirón et al. 1992) and is involved in protecting cells from stresses such as oxidative stress caused by treatment with free chlorine (Dukan and Touati 1996b) and hydrogen peroxide (Dukan and Touati 1996b, Martinez and Kolter 1997). *katE* is a monofunctional catalase HPII, which can decompose hydrogen peroxide into water and oxygen. Although no literature reports hydrogen peroxide formation during chlorine disinfection, other studies also reported up-regulation of *katE* and *katG* upon exposure to chlorine (Dukan and Touati 1996b, Wang et al. 2009).

Heat shock proteins—*htpG* is the *E. coli* homolog of the ubiquitous HSP90 protein family, and can bind to the heat shock alternative sigma factor σ^{32} and participate in folding of newly synthesized proteins under heat shock conditions (Thomas and Baneyx 2000). *grpE* belongs to a chaperone system that can convert misfolded proteins to their nascent forms (Sharma et al. 2010). Heat shock proteins were up-regulated during chlorine disinfection, probably because many chlorine reactions are exothermic and produce heat (Panasenko et al. 2013). Alternatively, there could be overlap in metabolic adaptations in response to insults like heat, detergent, and disinfectant, if they all respond to a signal of abnormal or unfolded proteins (Rajagopal et al. 2002).

Effects of chlorine on the various protein functional categories in normal and starved cells were analyzed using GO annotation (Figure 4 A). The bars above zero represent the percentage of proteins that were up-regulated within each protein functional group, while the bars below zero represent the percentage of proteins that were down-regulated within each functional group. In the GO annotation comparison between normal and starved cells during chlorine disinfection, starved cells had higher percentage of up-regulated proteins in the “response to stimulus” category than normal cells (Figure 4 A). Most of the proteins in this category are related to various types of stress response.

3.4 Monochloramine Disinfection on Normal and Starved cells

Similar to chlorine disinfection, starvation also promoted the expression of some proteins belonging to stress regulator (*hupB*) and heat shock proteins (*grpE*, Table 2). The trend seen in chlorine disinfection (Table 1) was also seen in monochloramine disinfection (Table 2), suggesting that starvation also prepared *E. coli* cells to monochloramine disinfection. Noticeably, thioredoxin/glutathione peroxidase (*btuE*) was up-regulated 8.5 fold in starved cells during monochloramine disinfection, suggesting involvement of reduced glutathione (GSH) in resistance to monochloramine. In the GO annotation comparison on monochloramine disinfection, starved cells exhibited higher percentage of up-regulated proteins in the “response to stimulus” category than normal cells (Figure 4 B), a trend that was also seen in chlorine disinfection (Figure 4 A).

3.5 Chlorine and Monochloramine Disinfection on Normal Cells

The genes that were up-regulated under both chlorine and monochloramine disinfection are believed to facilitate disinfection tolerance (Table 3). These up-regulated proteins were mainly in the protein functional groups of stress regulator, oxidative stress response protein, and osmotic stress protein. GO annotation shows that more proteins of “cellular component

organization or biogenesis” were up-regulated in normal cells during chlorine disinfection than during monochloramine disinfection (Figure 4 C). Most of the up-regulated proteins in this category were related to cell wall synthesis, suggesting chlorine might cause more cell wall damage than monochloramine due to its higher oxidative capacity. This may partially explain why there was no shoulder phase in chlorine disinfection (Figure 1).

Disinfection caused increased expression of glyoxalase 3 (*hchA*), 6 fold during chlorine disinfection and 10 fold during monochloramine disinfection (Table S2). One major function of glyoxalase 3 is to detoxify methylglyoxal (MG) by converting it into D-lactate in bacteria and fungi (Hasim et al. 2014, Misra et al. 1995). MG is an electrophile and can cause cell death by interacting with the nucleophilic centers of macromolecules such as DNA. MG only accumulates inside cells when the production outstrips the detoxification capacities (Ferguson et al. 1998). In *E. coli*, MG may be synthesized when the inhibition of glyceraldehyde-3-P dehydrogenase (*gapA*) cause accumulation of dihydroxyacetone phosphate (Ferguson et al. 1998). In this study, *gapA* was down-regulated during chlorination and monochloramination on normal cells (Table S2), potentially predisposing cells for MG production. Given the down-regulation of *gapA* and the up-regulation of *hchA*, we speculate that the balance between the production and detoxification of MG in cells may determine disinfection resistance of *E. coli* cells. No literature specifically studied whether chlorination or monochloramination of bacterial cells would yield MG, but it is known that MG can be excreted out of cells and that MG was detected in water during disinfection using ozone and chlorine, and is expected to be found during disinfection by other oxidants (Amy et al. 2000).

Protein-protein interactions among the shared, differentially expressed proteins were studied (Figure 5). The network nodes are proteins and the black lines indicate the presence of co-expression evidence. Co-expression of proteins suggests their involvement in the same metabolic pathway or share of synergistic functions (van Noort et al. 2003). For chlorine disinfection, oxidative stress response protein *katE* (up-regulated 2.3 and 4.0 fold in normal and starved cells) and heat shock protein *htpG* (up-regulated 2.6 and 7.5 fold in normal and starved cells) co-expressed, respectively, with proteins in pentose phosphate pathway (transketolase 2, *tktB*, up-regulated 5.7 and 5.7 fold in normal and starved cells) and in glycolysis (2,3-bisphosphoglycerate-dependent phosphoglycerate mutase, *gpmA*, up-regulated 2.3 and 5.6 fold in normal and starved cells, Figure 5A), which were both up-regulated under starvation (Figure 3). Like in chlorine disinfection, *gpmA* (up-regulated 2.9 and 5.89 fold in normal and starved cells) was among the co-expressed proteins with *htpG* (up-regulated 2.4 and 5.8 fold in normal and starved cells) during monochloramine disinfection (Figure 5B).

Results from this proteomic study are in general agreement with those from previous transcriptomics studies. Heat shock proteins (*htpG*) and oxidative stress response proteins (*katG* and *wrbA*) were detected to be up-regulated in chlorine and monochloramine disinfection using microarray (Berry et al. 2010, Wang et al. 2009). However, several up-regulated genes that were detected using transcriptomics were not detected in this study, for example genes related to iron-sulfur cluster assembly, cysteine biosynthesis and antibiotic resistance (Berry et al. 2010, Wang et al. 2009). This is likely due to the fundamental

differences between the transcriptomic and proteomic approaches. mRNA transcripts do not always directly correlate with the expression levels of the encoded proteins. During protein synthesis, the polypeptide chains translated from mRNA at the ribosome may undergo posttranslational modifications, such as folding and cutting (Cox and Mann 2011). Compared to transcriptomics, proteomics can detect protein abundances, stabilities, turnover rates, posttranslational modification, and protein-protein interactions describing molecular responses at the protein level. Because of these differences, proteomics and transcriptomics may lead to different results for the same samples (Drexler et al. 2012, Durban et al. 2013, Nikinmaa et al. 2013).

4. CONCLUSIONS

Proteomic analyses show that proteomic responses to starvation caused the up-regulation of proteins related to stress regulator, oxidative stress and osmotic stress, and prepared *E. coli* cells to survive disinfection by chlorine and monochloramine. This study further reveals that glutathione and methylglyoxal metabolisms may be important for bacterial defense to chlorine-based disinfection. These results improve our understanding of how starvation may prepare bacterial cells to survive low level chlorine or monochloramine in DWDSs. More studies on the function of the up-regulated proteins detected in this study can further elucidate the mechanisms of bacterial survival and re-growth in DWDSs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

5. ACKNOWLEDGEMENTS

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REFERENCES

- Albrich JM, Hurst JK. Oxidative Inactivation of Escherichia-Coli by Hypochlorous Acid - Rates and Differentiation of Respiratory from Other Reaction Sites. *FEBS Letters*. 1982; 144(1):157–161. [PubMed: 6286355]
- Ali Azam T, Iwata A, Nishimura A, Ueda S, Ishihama A. Growth phase-dependent variation in protein composition of the Escherichia coli nucleoid. *J Bacteriol*. 1999; 181(20):6361–6370. [PubMed: 10515926]
- Almirón M, Link AJ, Furlong D, Kolter R. A novel DNA-binding protein with regulatory and protective roles in starved Escherichia coli. *Genes Dev*. 1992; 6(12B):2646–2654. [PubMed: 1340475]
- Amy, G.; Bull, R.; Craun, GF.; Pegram, RA.; Siddiqui, M. *Disinfectants and Disinfectant By-Products*. Geneva: World Health Organization; 2000.
- Arenas FA, Díaz WA, Leal CA, Pérez-Donoso JM, Imlay JA, Vásquez CC. The Escherichia coli btuE gene, encodes a glutathione peroxidase that is induced under oxidative stress conditions. *Biochem Biophys Res Commun*. 2010; 398(4):690–694. [PubMed: 20621065]
- Berry D, Xi CW, Raskin L. Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology*. 2006; 17(3):297–302. [PubMed: 16701992]
- Berry D, Xi C, Raskin L. Effect of Growth Conditions on Inactivation of Escherichia coli with Monochloramine. 2008

- Berry D, Holder D, Xi C, Raskin L. Comparative transcriptomics of the response of *Escherichia coli* to the disinfectant monochloramine and to growth conditions inducing monochloramine resistance. *Water Res.* 2010; 44(17):4924–4931. [PubMed: 20692677]
- Cherchi C, Gu AZ. Effect of bacterial growth stage on resistance to chlorine disinfection. *Water Science and Technology.* 2011; 64(1):7–13. [PubMed: 22053451]
- Cho M, Kim J, Kim JY, Yoon J, Kim JH. Mechanisms of *Escherichia coli* inactivation by several disinfectants. *Water Res.* 2010; 44(11):3410–3418. [PubMed: 20427068]
- Cox J, Mann M. Quantitative, high-resolution proteomics for data-driven systems biology. *Annu Rev Biochem.* 2011; 80:273–299. [PubMed: 21548781]
- Dong T, Yu R, Schellhorn H. Antagonistic regulation of motility and transcriptome expression by RpoN and RpoS in *Escherichia coli*. *Molecular Microbiology.* 2011; 79(2):375–386. [PubMed: 21219458]
- Drexler HC, Ruhs A, Konzer A, Mendler L, Bruckskotten M, Looso M, Günther S, Boettger T, Krüger M, Braun T. On marathons and Sprints: an integrated quantitative proteomics and transcriptomics analysis of differences between slow and fast muscle fibers. *Mol Cell Proteomics.* 2012; 11(6):M111.010801. [PubMed: 22210690]
- Dukan S, Touati D. Hypochlorous acid stress in *Escherichia coli*: Resistance, DNA damage, and comparison with hydrogen peroxide stress. *Journal of Bacteriology.* 1996a; 178(21):6145–6150. [PubMed: 8892812]
- Dukan S, Touati D. Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. *J Bacteriol.* 1996b; 178(21):6145–6150. [PubMed: 8892812]
- Durban J, Pérez A, Sanz L, Gómez A, Bonilla F, Rodríguez S, Chacón D, Sasa M, Angulo Y, Gutiérrez JM, Calvete JJ. Integrated "omics" profiling indicates that miRNAs are modulators of the ontogenetic venom composition shift in the Central American rattlesnake, *Crotalus simus simus*. *BMC Genomics.* 2013; 14:234. [PubMed: 23575160]
- Eaton, AD.; Clesceri, LS.; Rice, EW.; Greenberg, AE. *Standard Methods for the Examination of Water and Wastewater.* Washington, DC: American Public Health Association; 2005.
- Fahey RC, Brown WC, Adams WB, Worsham MB. Occurrence of Glutathione in Bacteria. *Journal of Bacteriology.* 1978; 133(3):1126–1129. [PubMed: 417060]
- Falkinham JO, Norton CD, LeChevallier MW. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other *Mycobacteria* in drinking water distribution systems. *Appl Environ Microbiol.* 2001; 67(3):1225–1231. [PubMed: 11229914]
- Ferguson GP, Totemeyer S, MacLean MJ, Booth IR. Methylglyoxal production in bacteria: suicide or survival? *Archives of Microbiology.* 1998; 170(4):209–219. [PubMed: 9732434]
- Hasim S, Hussin NA, Alomar F, Bidasee KR, Nickerson KW, Wilson MA. A Glutathione-independent Glyoxalase of the DJ-1 Superfamily Plays an Important Role in Managing Metabolically Generated Methylglyoxal in *Candida albicans*. *Journal of Biological Chemistry.* 2014; 289(3):1662–1674. [PubMed: 24302734]
- Holder D, Berry D, Dai DJ, Raskin L, Xi CW. A dynamic and complex monochloramine stress response in *Escherichia coli* revealed by transcriptome analysis. *Water Research.* 2013; 47(14):4978–4985. [PubMed: 23866139]
- Hurst JK, Barrette WC, Michel BR, Rosen H. Hypochlorous Acid and Myeloperoxidase-Catalyzed Oxidation of Iron-Sulfur Clusters in Bacterial Respiratory Dehydrogenases. *European Journal of Biochemistry.* 1991; 202(3):1275–1282. [PubMed: 1662610]
- Hwang C, Ling F, Andersen GL, LeChevallier MW, Liu WT. Microbial community dynamics of an urban drinking water distribution system subjected to phases of chloramination and chlorination treatments. *Appl Environ Microbiol.* 2012; 78(22):7856–7865. [PubMed: 22941076]
- Jacangelo JG, Olivieri VP, Kawata K. Oxidation of Sulfhydryl-Groups by Monochloramine. *Water Research.* 1987; 21(11):1339–1344.
- Jenkins DE, Schultz JE, Matin A. Starvation-induced cross protection against heat or H₂O₂ challenge in *Escherichia coli*. *J Bacteriol.* 1988; 170(9):3910–3914. [PubMed: 3045081]
- Jenkins DE, Chaisson SA, Matin A. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. *J Bacteriol.* 1990; 172(5):2779–2781. [PubMed: 2185233]

- Jung IL, Physo KH, Kim IG. RpoS-mediated growth-dependent expression of the *Escherichia coli* tkt genes encoding transketolases isoenzymes. *Current Microbiology*. 2005; 50(6):314–318. [PubMed: 15968503]
- Kar S, Edgar R, Adhya S. Nucleoid remodeling by an altered HU protein: reorganization of the transcription program. *Proc Natl Acad Sci U S A*. 2005; 102(45):16397–16402. [PubMed: 16258062]
- Larson MA, Marinas BJ. Inactivation of *Bacillus subtilis* spores with ozone and monochloramine. *Water Res*. 2003; 37(4):833–844. [PubMed: 12531265]
- Lechevallier MW, Schulz W, Lee RG. Bacterial Nutrients in Drinking-Water. *Applied and Environmental Microbiology*. 1991; 57(3):857–862. [PubMed: 2039235]
- LeChevallier MW, Welch NJ, Smith DB. Full-scale studies of factors related to coliform regrowth in drinking water. *Appl Environ Microbiol*. 1996; 62(7):2201–2211. [PubMed: 8779557]
- Liu H, Sadygov RG, Yates JR. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem*. 2004; 76(14):4193–4201. [PubMed: 15253663]
- Martínez-Gómez K, Flores N, Castañeda HM, Martínez-Batallar G, Hernández-Chávez G, Ramírez OT, Gosset G, Encarnación S, Bolívar F. New insights into *Escherichia coli* metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. *Microb Cell Fact*. 2012; 11:46. [PubMed: 22513097]
- Martinez A, Kolter R. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. *J Bacteriol*. 1997; 179(16):5188–5194. [PubMed: 9260963]
- Mckenna SM, Davies KJA. The Inhibition of Bacterial-Growth by Hypochlorous Acid - Possible Role in the Bactericidal Activity of Phagocytes. *Biochemical Journal*. 1988; 254(3):685–692. [PubMed: 2848494]
- Misra K, Banerjee AB, Ray S, Ray M. Glyoxalase-III from *Escherichia coli* - a single novel enzyme for the conversion of methylglyoxal into D-lactate without reduced glutathione. *Biochemical Journal*. 1995; 305:999–1003. [PubMed: 7848303]
- Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M. KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res*. 2007; 35(Web Server issue):W182–W185. [PubMed: 17526522]
- Nandakumar R, Espirito Santo C, Madayiputhiya N, Grass G. Quantitative proteomic profiling of the *Escherichia coli* response to metallic copper surfaces. *Biometals*. 2011; 24(3):429–444. [PubMed: 21384090]
- Nikinmaa M, McCairns RJ, Nikinmaa MW, Vuori KA, Kanerva M, Leinonen T, Primmer CR, Merilä J, Leder EH. Transcription and redox enzyme activities: comparison of equilibrium and disequilibrium levels in the three-spined stickleback. *Proc Biol Sci*. 2013; 280(1755):20122974. [PubMed: 23363636]
- Oberto J, Nabti S, Jooste V, Mignot H, Rouviere-Yaniv J. The HU regulon is composed of genes responding to anaerobiosis, acid stress, high osmolarity and SOS induction. *PLoS One*. 2009; 4(2):e4367. [PubMed: 19194530]
- Panasenko OM, Gorudko IV, Sokolov AV. Hypochlorous acid as a precursor of free radicals in living systems. *Biochemistry-Moscow*. 2013; 78(13):1466–1489. [PubMed: 24490735]
- Pohl S, Tu WY, Aldridge PD, Gillespie C, Hahne H, Mader U, Read TD, Harwood CR. Combined proteomic and transcriptomic analysis of the response of *Bacillus anthracis* to oxidative stress. *Proteomics*. 2011; 11(15):3036–3055. [PubMed: 21726052]
- Rajagopal S, Sudarsan N, Nickerson KW. Sodium dodecyl sulfate hypersensitivity of clpP and clpB mutants of *Escherichia coli*. *Applied and Environmental Microbiology*. 2002; 68(8):4117–4121. [PubMed: 12147516]
- Rangel DEN. Stress induced cross-protection against environmental challenges on prokaryotic and eukaryotic microbes. *World J Microbiol Biotechnol*. 2011; 27:1281–1296. [PubMed: 25187127]
- Saby S, Leroy P, Block JC. *Escherichia coli* resistance to chlorine and glutathione synthesis in response to oxygenation and starvation. *Applied and Environmental Microbiology*. 1999; 65(12):5600–5603. [PubMed: 10584025]

- Sharma SK, De los Rios P, Christen P, Lustig A, Goloubinoff P. The kinetic parameters and energy cost of the Hsp70 chaperone as a polypeptide unfoldase. *Nat Chem Biol.* 2010; 6(12):914–920. [PubMed: 20953191]
- Stewart MH, Olson BH. Impact of Growth-Conditions on Resistance of *Klebsiella- Pneumoniae* to Chloramines. *Applied and Environmental Microbiology.* 1992; 58(8):2649–2653. [PubMed: 1514811]
- Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguéz P, Doerks T, Stark M, Muller J, Bork P, Jensen LJ, von Mering C. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res.* 2011; 39(Database issue):D561–D568. [PubMed: 21045058]
- Thomas JG, Baneyx F. ClpB and HtpG facilitate de novo protein folding in stressed *Escherichia coli* cells. *Mol Microbiol.* 2000; 36(6):1360–1370. [PubMed: 10931286]
- Tong Z, Tao R, Jiang W, Li J, Zhou L, Tian Y, Ni L. In vitro study of the properties of *Streptococcus mutans* in starvation conditions. *Arch Oral Biol.* 2011; 56(11):1306–1311. [PubMed: 21704301]
- van Noort V, Snel B, Huynen MA. Predicting gene function by conserved co-expression. *Trends Genet.* 2003; 19(5):238–242. [PubMed: 12711213]
- Virto R, Mañas P, Álvarez I, Condon S, Raso J. Membrane Damage and Microbial Inactivation by Chlorine in the Absence and Presence of a Chlorine-Demanding Substrate. 2005
- Wang H, Masters S, Edwards MA, Falkinham JO, Pruden A. Effect of disinfectant, water age, pipe materials on bacterial and eukaryotic community structure in drinking water biofilm. *Environ Sci Technol.* 2014; 48(3):1426–1435. [PubMed: 24401122]
- Wang S, Deng K, Zaremba S, Deng X, Lin C, Wang Q, Tortorello ML, Zhang W. Transcriptomic response of *Escherichia coli* O157:H7 to oxidative stress. *Appl Environ Microbiol.* 2009; 75(19): 6110–6123. [PubMed: 19666735]
- Watters SK, Pyle BH, Lechevallier MW, Mcfeters GA. Enumeration of *Enterobacter-Cloacae* after Chloramine Exposure. *Applied and Environmental Microbiology.* 1989; 55(12):3226–3228. [PubMed: 2619309]
- Winter J, Ilbert M, Graf PCF, Ozcelik D, Jakob U. Bleach Activates a Redox-Regulated Chaperone by Oxidative Protein Unfolding. *Cell.* 2008; 135(4):691–701. [PubMed: 19013278]
- Zhang XJ, Chen C, Wang Y. Synergetic Inactivation of Microorganisms in Drinking Water by Short-term Free Chlorination and Subsequent Monochloramination. *Biomed. Environ. Sci.* 2007; 20(5): 373–380. [PubMed: 18188988]

Highlights

- Starved cells exhibited higher tolerance than normal cells during disinfection.
- Proteins up-regulated under starvation were further up-regulated in disinfection.
- Proteins involved in stress regulation and responses are key to cross-protection.
- Glutathione and methylglyoxal metabolisms are potentially important.

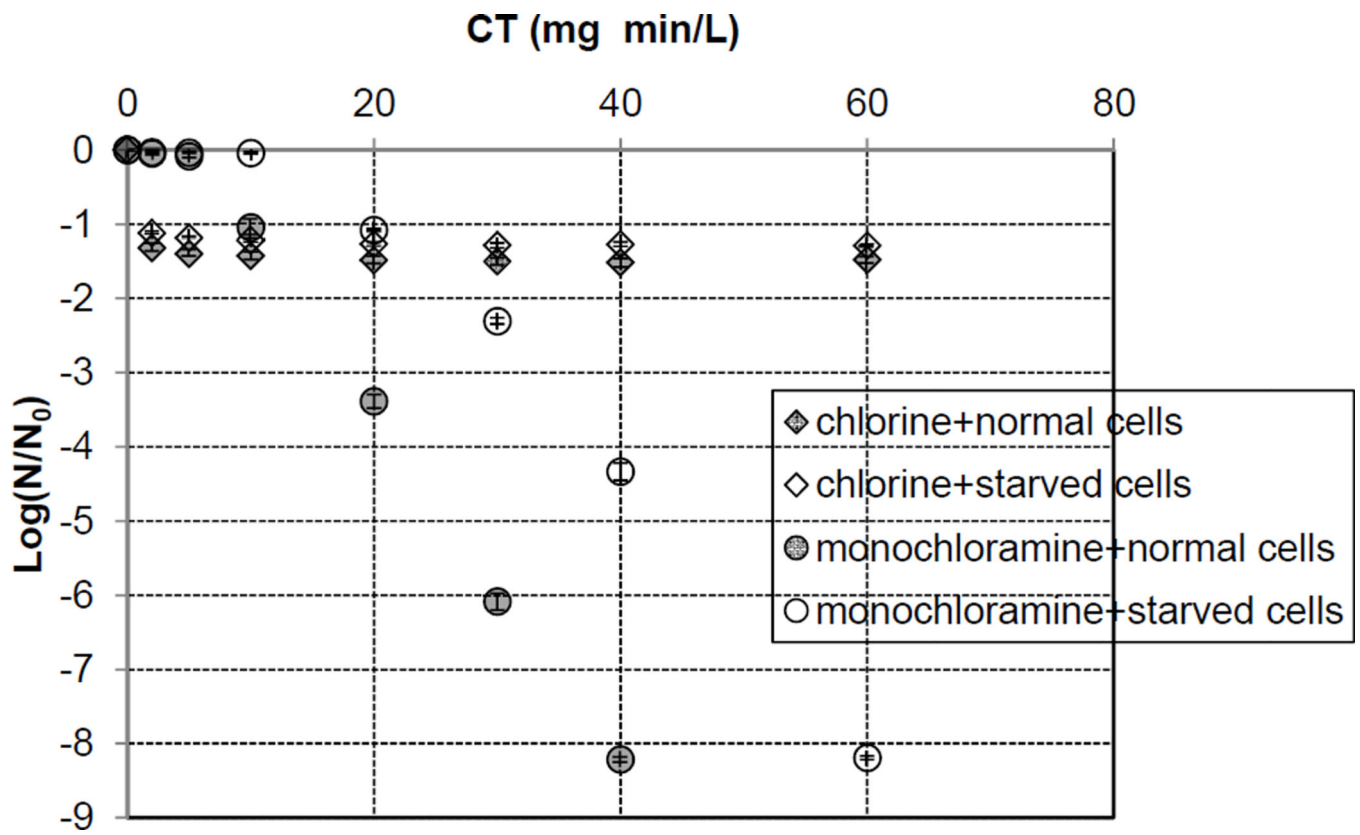


Figure 1. The inactivation curves of normal and starved *E. coli* cells with chlorine and monochloramine. N and N_0 are the cell count at time t and time zero, respectively. The error bars represent the standard error from triplicate inactivation experiments.

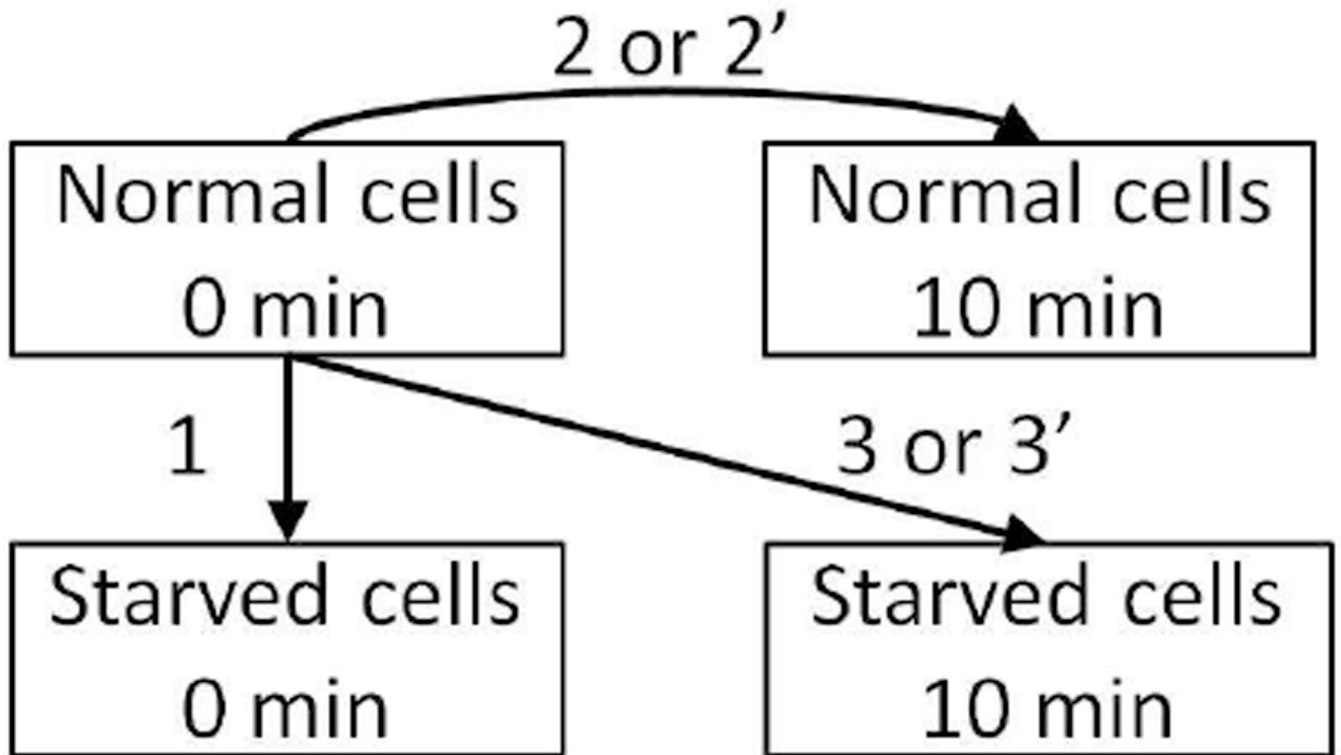


Figure 2.

Comparison scheme used in proteomic analyses. Arrows originate from reference proteomes and point to treatment proteomes. Comparison 1 is for starvation, while Comparisons 2 and 3 are for chlorine disinfection (chlorination) and Comparisons 2' and 3' for monochloramine disinfection (chloramination).

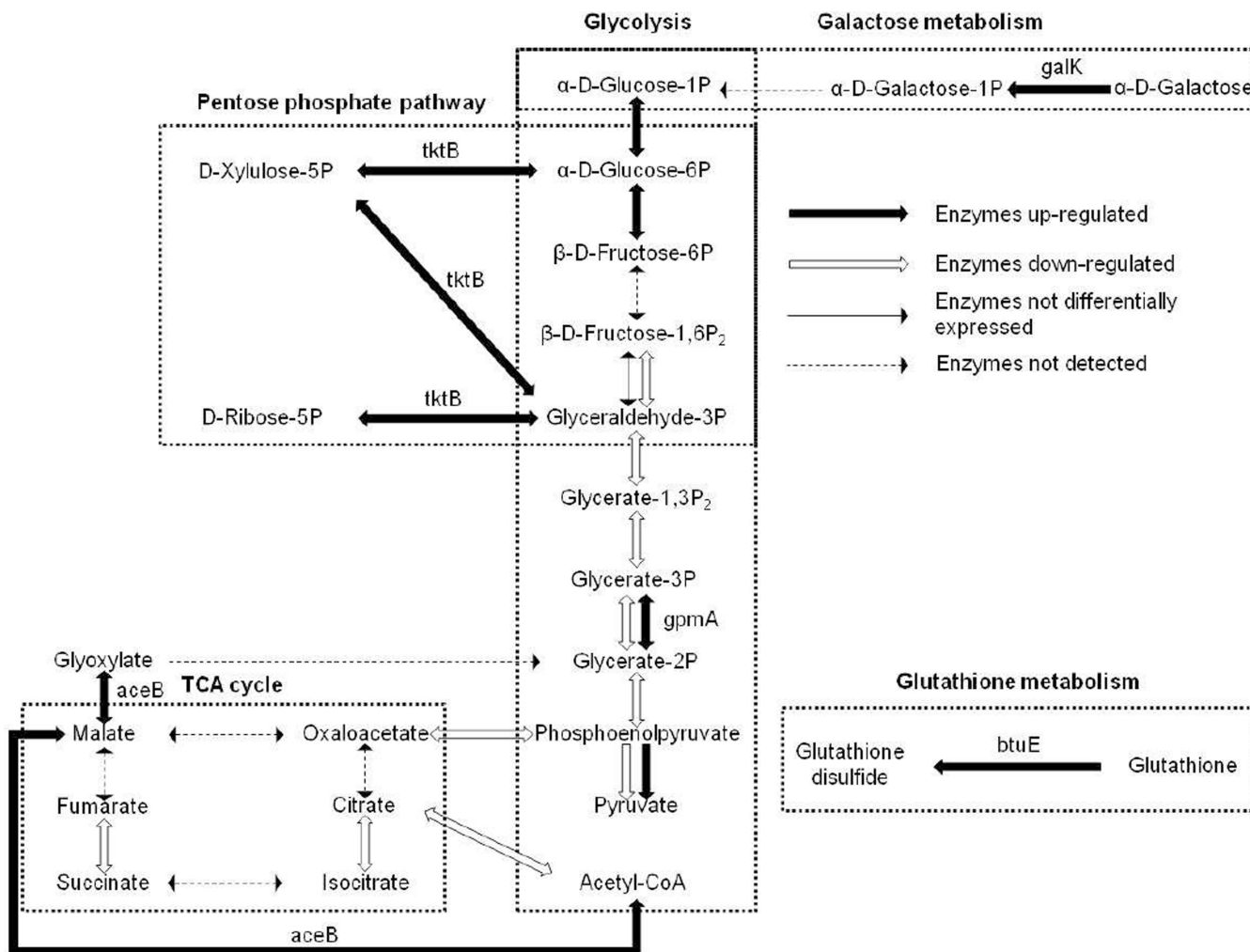


Figure 3. Metabolic reactions that were catalyzed by enzymes up-regulated (bold arrow), enzymes down-regulated (outlined arrow), enzymes not differentially expressed (thin arrow), and by enzymes undetected (dotted arrow) upon starvation.

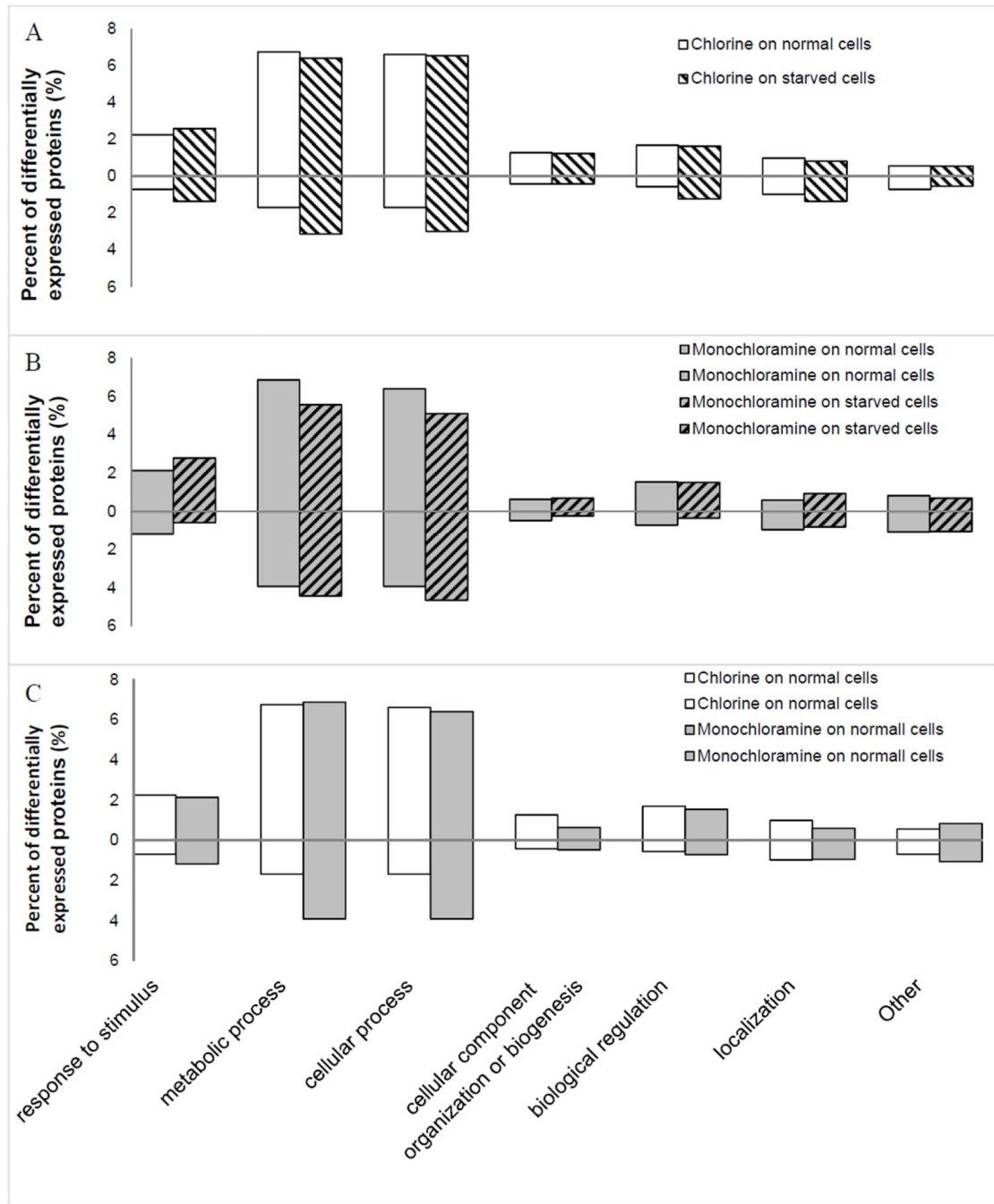


Figure 4. GO annotation comparison of differentially expressed proteins (A) during chlorine disinfection on normal and starved cells; (B) during monochloramine disinfection on normal and starved cells; and (C) during chlorine and monochloramine disinfection of normal cells.

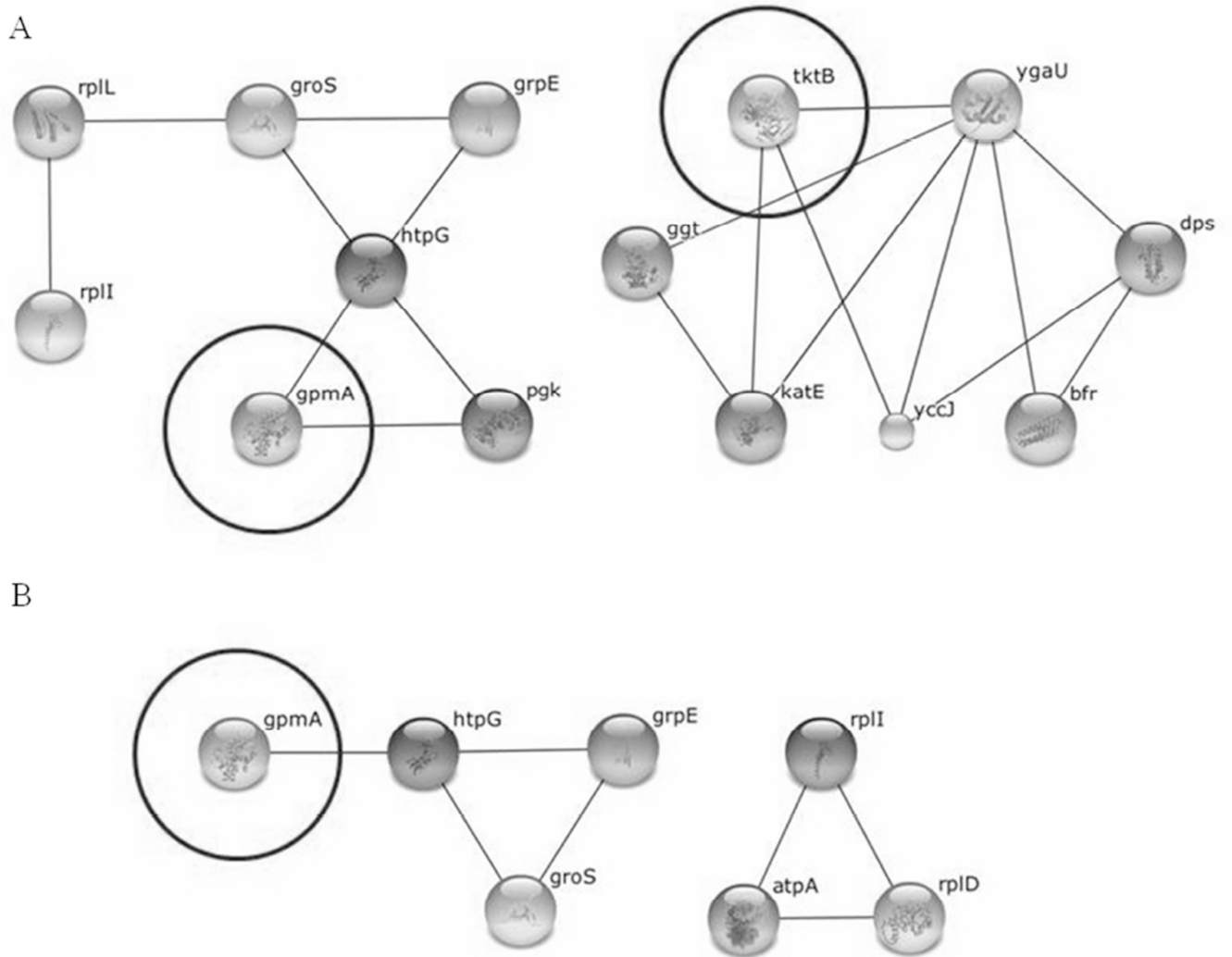


Figure 5. Proteins (network nodes) that were co-expressed (indicated by black lines) in normal and starved cells during chlorine (A) and monochloramine (B) disinfection. Proteins circled were also up-regulated under starvation.

Table 1Differentially expressed proteins in normal and starved *E. coli* cells during chlorine disinfection.

Gene	Fold change			
	Normal 10 min (Comparison 2)	Starved 0 min (Comparison 1)	Starved 10 min (Comparison 3)	
<i>Stress regulator</i>				
hupB	HU, DNA-binding transcriptional regulator, beta subunit	3.9	6.0	6.9
<i>Oxidative stress response protein</i>				
dps	Fe-binding and storage protein	1.5	2.0	6.1
katE	Hydroperoxidase HP11 (III) (catalase)	2.3	3.2	4.0
<i>Heat shock protein</i>				
htpG	molecular chaperone HSP90 family	2.6	3.7	7.5
grpE	heat shock protein	2.3	2.1	13.0

Table 2Differentially expressed proteins in normal and starved *E. coli* cells during monochloramine disinfection.

Gene		Fold change		
		Normal 10 min (Comparison 2)	Starved 0 min (Comparison 1)	Starved 10 min (Comparison 3')
<i>Stress regulator</i>				
hupB	HU, DNA-binding transcriptional regulator, beta subunit	0.9	6.0	14.3
<i>Oxidative stress response protein</i>				
btuE	Thioredoxin/glutathione peroxidase	0.3	2.8	8.5
<i>Heat shock protein</i>				
grpE	Heat shock protein	0.6	2.1	6.3
htpG	molecular chaperone HSP90 family	2.4	3.7	5.8

Table 3Differentially expressed proteins in normal *E. coli* cells under chlorine and monochloramine disinfection.

Gene	Fold change		
	Normal 10 min Chlorine	Normal 10 min Monochloramine	
<i>Stress regulator</i>			
ospC	stress protein, member of the CspA-family	4.7	2.1
yajQ	Phi6 host factor; binds ATP and GTP	6.0	16.3
<i>Oxidative stress response protein</i>			
wrbA	NAD(P)H:quinone oxidoreductase	2.1	2.2
ahpF	alkyl hydroperoxide reductase, F52a subunit, FAD/NAD(P)-binding	10.5	4.6
uspF	stress-induced protein, ATP-binding protein	4.1	2.1
katG	catalase-peroxidase HPI, heme b-containing	4.5	2.6
<i>Osmotic stress protein</i>			
yciF	predicted rubrerythrin/ferritin-like metal-binding protein	5.5	10
<i>Enzymes related to the production and detoxification of MG</i>			
hchA	glyoxalase 3	6.0	10.0
gapA	glyceraldehyde-3-P dehydrogenase A	0.6*	0.2

* gapA was down-regulated – albeit marginally significant – under this condition.