Mass spectrometry-based profiling of the carbon starved *Escherichia coli* proteome reveals upregulation of stress-inducible pathways implicated in biofilm formation and antibiotic resistance.

Rakhan Aimbetov, Vasily Ogryzko

Starvation is a complex adaptive response to insufficiency of nutrients that has been known to implicate a number of stress networks, and modulate pathogenicity and antibiotic resistance in bacteria. However, naturally occurring abrupt elimination of nutrients and prolonged periods of their complete absence, e.g. when bacteria are placed in natural or artificial water reservoirs, are qualitatively different from in-culture late stationary phase energy source diminution. Despite the obvious importance of proteomic investigation of bacteria exposed to nutrient deficiency, no comprehensive study on the subject has been published. In order to address the said shortage of knowledge, we decided to quantitatively look into the proteome-level alterations elicited by the complete lack of nutrients that constitute a viable source of carbon, i.e. carbon starvation, in the *Escherichia coli* HT115-derived SLE1 strain cells using the combination of label-free and SILAC-based proteomics. As a result, we obtained protein ratios for 1,757 and 1,241 protein groups for each technique respectively, 2D-annotated the quantifiable proteins present in both datasets, identified over- and underrepresented Gene Ontology terms, and isolated protein groups ≥2-fold up- and downregulated in response to carbon starvation (44 and 36 protein groups respectively). We observed upregulation of proteins implicated in various stress-related networks, most notably those that constitute the Gene Ontology term '*Biological adhesion*', as well as various terms related to stress. Additionally, we identified several uncharacterized proteins, and our report is the first to ascribe them to a stress-induced proteome. Our data are available via ProteomeXchange with identifier PXD003255 and DOI:10.6019/PXD003255.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1572v1 | CC-BY 4.0 Open Access | rec: 8 Dec 2015, publ: 8 Dec 2015

Mass spectrometry-based profiling of the carbon starved *Escherichia coli* proteome reveals upregulation of stress-inducible pathways implicated in biofilm formation and antibiotic resistance

Rakhan Aimbetov[1], Vasily Ogryzko[1]

[1] CNRS UMR 8126, Institut Gustave Roussy, Villejuif, France

Corresponding author:

Rakhan Aimbetov

114 rue Edouard Vaillant, Villejuif, 94805, France

E-mail address: r.aimbetov@gmail.com

ABSTRACT

Starvation is a complex adaptive response to insufficiency of nutrients that has been known to implicate a number of stress networks, and modulate pathogenicity and antibiotic resistance in bacteria. However, naturally occurring abrupt elimination of nutrients and prolonged periods of their complete absence, e.g. when bacteria are placed in natural or artificial water reservoirs, are qualitatively different from in-culture late stationary phase energy source diminution. Despite the obvious importance of proteomic investigation of bacteria exposed to nutrient deficiency, no comprehensive study on the subject has been published. In order to address the said shortage of knowledge, we decided to quantitatively look into the proteome-level alterations elicited by the complete lack of nutrients that constitute a viable source of carbon, i.e. carbon starvation, in the *Escherichia coli* HT115-derived SLE1 strain cells using the combination of label-free and SILAC-based proteomics. As a result, we obtained protein ratios for 1,757 and 1,241 protein groups for each technique respectively, 2D-annotated the quantifiable proteins present in both datasets, identified over- and underrepresented Gene Ontology terms, and isolated protein groups ≥2-fold up- and downregulated in response to carbon starvation (44 and 36 protein groups respectively). We observed upregulation of proteins implicated in various stress-related networks, most notably those that constitute the Gene Ontology term *'Biological adhesion'*, as well as various terms related to stress. Additionally, we identified several uncharacterized proteins, and our report is the first to ascribe them to a stress-induced proteome. Our data are available via ProteomeXchange with identifier PXD003255 and DOI:10.6019/PXD003255.

INTRODUCTION

Continuous development of mass spectra acquisition tools, in parallel with sophisticated bioinformatical data analysis environments, has ensured the rising popularity of mass spectrometry as a powerful technology for protein identification and quantification. Stable isotope labeling by amino acids in culture (SILAC) is a preferred approach when it comes to quantitative evaluation of relative protein abundance based on metabolic incorporation of 'light' or 'heavy' versions of lysine and arginine into nascent polypeptides (Mann, 2014). The possibility of simultaneous sample manipulation, granted by the labeling, conveniently allows uniform processing eliminating the variation in preparation and analysis. Label-free quantification, on the other hand, is a cheaper and less laborious technique that has recently been getting more attention due to its virtual universality (Megger et al., 2013).

Bacterial starvation is a complex phenotype of growth retardation, reduced viability, and overall switching of cellular metabolic machinery to a more robust energy-saving mode in response to the stress of nutritional scarcity. Given the implication of stress-induced pathways in modulation of pathogenicity (Fang et al., 1992; Suh et al., 1999; Thompson et al., 2003) and antibiotic resistance (Nachin, Nannmark & Nyström, 2005; Petrosino et al., 2009; Nguyen et al., 2011; Poole, 2012; Bernier et al., 2013; Bokinsky et al., 2013; Prax & Bertram, 2014), the study of proteome-level alterations evoked by starvation possesses a significant theoretical and practical interest. However, upon having closely examined the available literature, we have to admit that the research which truly looks into the proteome of a nutrient-deprived bacterial cell is lacking. In order to appease the current shortage of data on proteomic peculiarities of a starved bacterial cell, we embarked on assessing the qualitative and quantitative changes imparted by carbon starvation on a bacterial proteome utilizing the combination of label-free and SILAC methodologies.

MATERIALS AND METHODS

Strain and culture conditions – We used HT115-derived *Escherichia coli* strain SLE1 auxotrophic for arginine and lysine. The cells were grown in M9 minimal medium (5.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl_2, 1 mM $MgSO_4$, 0.2% glucose, 0.01% thiamine), supplemented with 0.3 mM of either $^{12}C_6$-lysine/$^{12}C_6{}^{14}N_4$-arginine ('light') or $^{13}C_6$-

lysine/$^{13}C_6{}^{15}N_4$-arginine ('heavy') amino acids, at 37°C and 150 rpm. Carbon starvation was achieved by incubation of the cells in the medium devoid of amino acids and glucose for 48 hrs (Fig.S1).

Sample preparation – The cells were collected by centrifugation, washed once with cold PBS, and lysed in 1X LDS loading buffer (Novex). After estimation of protein concentration, equal quantities of protein, typically ≤400 µg, were processed in accordance with FASP protocol (Wiśniewski et al., 2009). Briefly, a necessary volume of a reduced protein sample, up to 30 µL, was mixed with 200 µL of 8 M urea in 0.1 M Tris-HCl pH 8.5 and loaded on 10 kDa cut-off spin filters (Millipore). Cysteines were alkylated by 50 mM iodoacetamide in the urea solution for 20 min in the dark. Protein digestion was performed by 15 ng/µL trypsin in 50 mM ammonium bicarbonate for 18 hrs at 37°C. Eluted peptides were desalted on Vivapure C18 micro spin columns (Sartorius Stedim Biotech), desiccated in SpeedVac and dissolved in 10 µL of LC buffer A (0.1% formic acid in water).

Mass spectra acquisition – LC/MS analysis was performed on EASY-nLC 1000 (Thermo Scientific) paired with Q Exactive quadrupole-orbitrap hybrid mass spectrometer (Thermo Scientific). The peptide mixture was separated on EASY-Spray 15 cm × 75 µm 3 µm 100Å C18 PepMap® reverse-phase column (Thermo Scientific) using 150 min three-step water-acetonitrile gradient (0-120 min, 5 → 35% LC buffer B (0.1% formic acid in acetonitrile); 120-140 min, 35 → 50%; 140-145 min, 50 → 90%; hold for 5 min) at 300 nL/min flow rate. The intensities of precursor ions were gauged in positive mode at scan range 400-2,000 m/z, resolution 70,000, automatic gain control (AGC) target 1E6, maximum injection time 100 ms, followed by forwarding 10 most intense ions of a spectrum for MS2 fragmentation and measurement at resolution 17,500, AGC target 5E4, maximum injection time 100 ms, isolation window 2 m/z with 30 sec dynamic exclusion.

Discovery analysis – Raw mass spectrometric data were analyzed by Proteome Discoverer v.1.4.0.288. MS2 spectra were searched against the *Escherichia coli* Swiss-Prot database using Mascot engine set for 10 ppm precursor mass and 0.02 Da fragment mass tolerances with 2 allowed missed cleavage sites. For labeled samples, the amino acid modifications were as follows: $^{13}C_6$-lysine (+6.020129 Da) and $^{13}C_6{}^{15}N_4$-arginine (+10.008269 Da) SILAC labels, methionine oxidation (+15.994915 Da) as dynamic, cysteine carbamidomethylation (+57.021464 Da) as static. For unlabeled samples: methionine oxidation and asparigine/glutamine deamidation (+0.984016 Da) as dynamic, cysteine carbamidomethylation as static. False discovery rate (FDR) was calculated using Percolator (Brosch et al., 2009) with 0.01 strict and 0.05 relaxed target cut-off values.

Protein quantification – Label-free comparative protein quantitation was carried out in Sieve v.2.1.377. Total ion current (TIC) alignment was done on 5-120 min segment with 2 min retention time (RT) shift limit. Framing was performed on a 400-2,000 m/z range with RT and m/z widths equal to 2.5 min and 10 ppm respectively while *'Frames from MS2 scans'* option was assigned a *TRUE* value. Protein IDs were imported from Proteome Discoverer. SILAC H/L ratios were determined using Proteome Discoverer's Precursor Ions Quantifier node with the experimental bias normalization based on at least 20 protein counts.

GO term enrichment – Two-dimensional annotation enrichment was done in Perseus v.1.5.0.9 (Cox & Mann, 2012). The lists of over- and underrepresented pathways were created using the functional annotation tool of DAVID Bioinformatic Resources 6.7 database (http://david.abcc.ncifcrf.gov/) (Huang, Sherman & Lempicki, 2009).

Data reposition – All raw files with the accompanying result output have been uploaded to ProteomeXchange Consortium repository (http://www.proteomexchange.org/) via PRIDE (Vizcaíno et al., 2013) with the dataset identifier PXD003255 and DOI:10.6019/PXD003255.

RESULTS

For brevity, henceforth in this paper the samples and proteins derived from unlabeled cells will be simply referred to as label-free or LFQ, e.g. label-free samples, LFQ proteins, while if originated from metabolically labeled cells they will be referred to as labeled or SILAC, e.g. labeled samples, SILAC proteins.

The goal of the present project was to quantify the qualitative changes, triggered and enhanced by starvation, of a cellular composition in relation to a proteome pertinent to cells exponentially growing in the late log-phase (OD_{600} 0.3). The combination of label-free- and SILAC- based quantification avenues permits the usage of these two methods as validators of each other, and consequently allows the confident identification of differentially regulated proteins and networks thereof while weeding out the inevitable 'flukes' in detection.

For practical reasons we define starvation as a state which develops in response to nutrient scarcity, and that is marked by decline in cellular growth, proliferation, and overall viability. The scientific literature provides conflicting information on the time required for *Escherichia coli* to achieve starvation. It seems, however, that the duration of deprivation largely depends on the strain under investigation and the conditions selected to elicit starvation. In order to tailor our conditions to the strain chosen, we undertook a series of direct plate count experiments in which a certain dilution of starved incubation culture was plated out onto LB-agar medium at defined time points with subsequent enumeration of the colonies grown. As a result we have learned that 48 hours is a threshold after which the cells start to lose their viability (data not shown). Accordingly, our workflow employed 48 hours starvation period for both LFQ and SILAC methodologies as optimal for our purposes.

The MS analysis of label-free samples resulted in 130,526 and 118,455 spectra for exponential and starved cells which matched 8,603 and 7,769 high-confidence peptides assigned to 1,602 and 1,437 protein groups (1,757 in total) respectively. For labeled samples, 125,220 spectra, matched with 6,037 high-confidence peptides, allowed to identify 1,241 protein groups.

Of 1,757 protein groups discovered by the label-free approach 1,128 were quantifiable (Table S1 and S2), whereas of 1,241 groups identified by the SILAC-based technique 1,087 were quantified (Table S3 and S4). Of the groups with calculated ratios 822 were present in both datasets (Fig.S2). The ratios of the protein groups common for both sets were $\log_2$- and *z*-transformed, and plotted for correlation analysis (Fig.1). The ratio distribution followed the Gaussian pattern, whereas the Pearson's correlation coefficient between the ratios, obtained using the two methods, was equal to 0.63 ($R^2 = 0.39$).

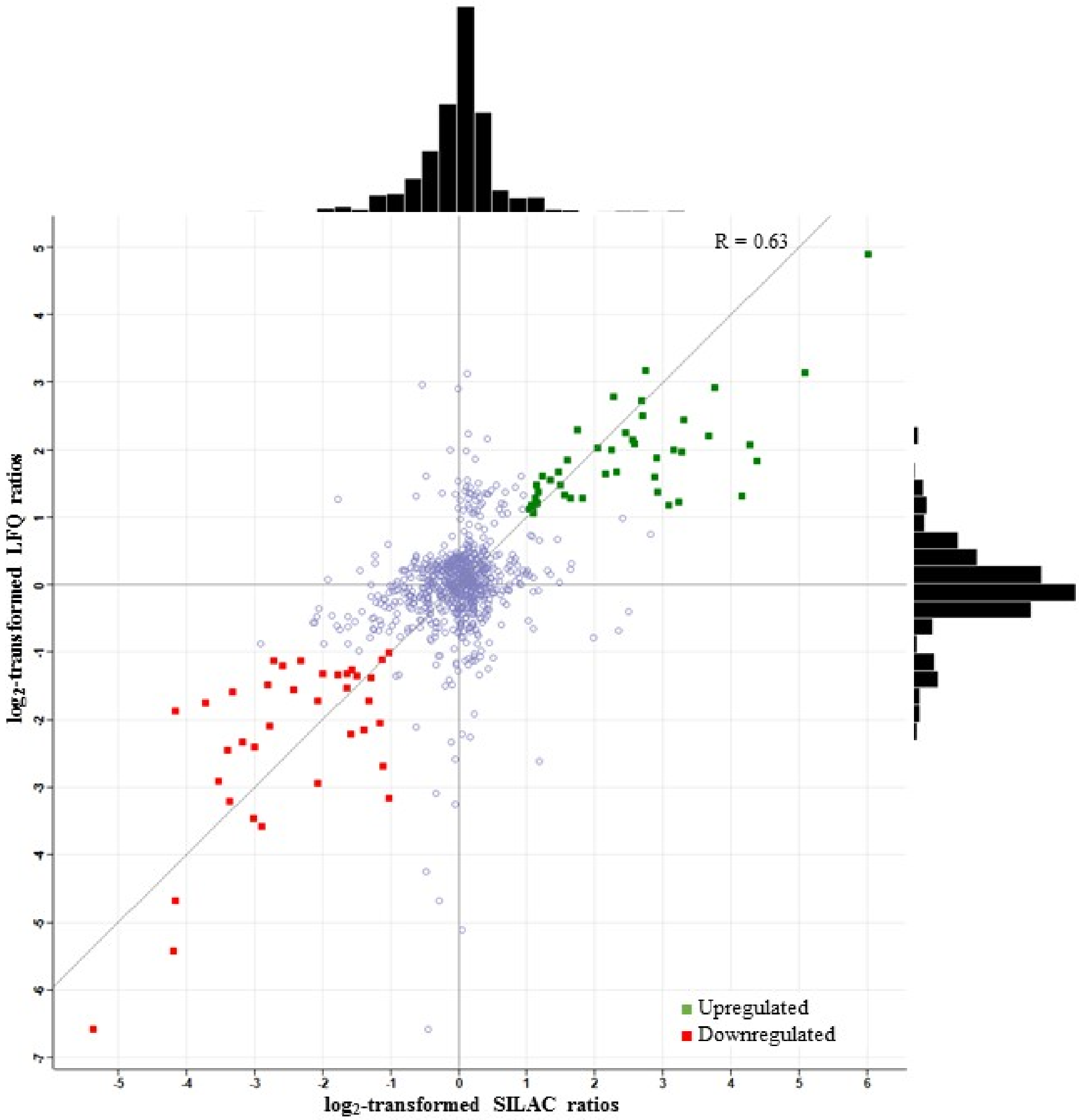

Figure1. Comparison and correlation analysis of log_2-transformed SILAC *(x)* and LFQ *(y)* ratios. *Histogram on top*, distribution of the protein ratios obtained by SILAC-based quantification (n = 822). *Histogram on the right*, distribution of the protein ratios obtained by label-free quantification (n = 822). *Scatter plot*, the protein ratios plotted against *x* and *y* axis (n = 822). *Green*, protein ratios > 1. *Red*, protein ratios < -1. *Blue*, protein ratios [-1, 1]. Pearson's correlation score R = 0.63 (R^2 = 0.39).

Gene Ontology (GO) is a comprehensive vocabulary of genes' and gene products' functional descriptions arranged into categories and terms (Ashburner et al., 2000). In order to establish what metabolic pathways were affected by the starvation we took advantage of Perseus's two-dimensional annotation feature (Cox & Mann, 2012) and compared LFQ and SILAC datasets isolating GO terms with the highest enrichment and correlation score (Table S5, Fig.2). As seen from the figure, the most overrepresented biological process GO term (GOBP) was *'Biological adhesion'* which is in line with numerous reports of various stresses converging on cellular adhesion in bacteria, as we will discuss in more detail in the next section. Conspicuous underrepresentation of *'Small ribosomal subunit'* and *'Large ribosomal subunit'* cellular compartment terms (GOCC) reflects the overall downshift in *de novo* protein synthesis and downregulation of ribosomal proteins in starved cells.

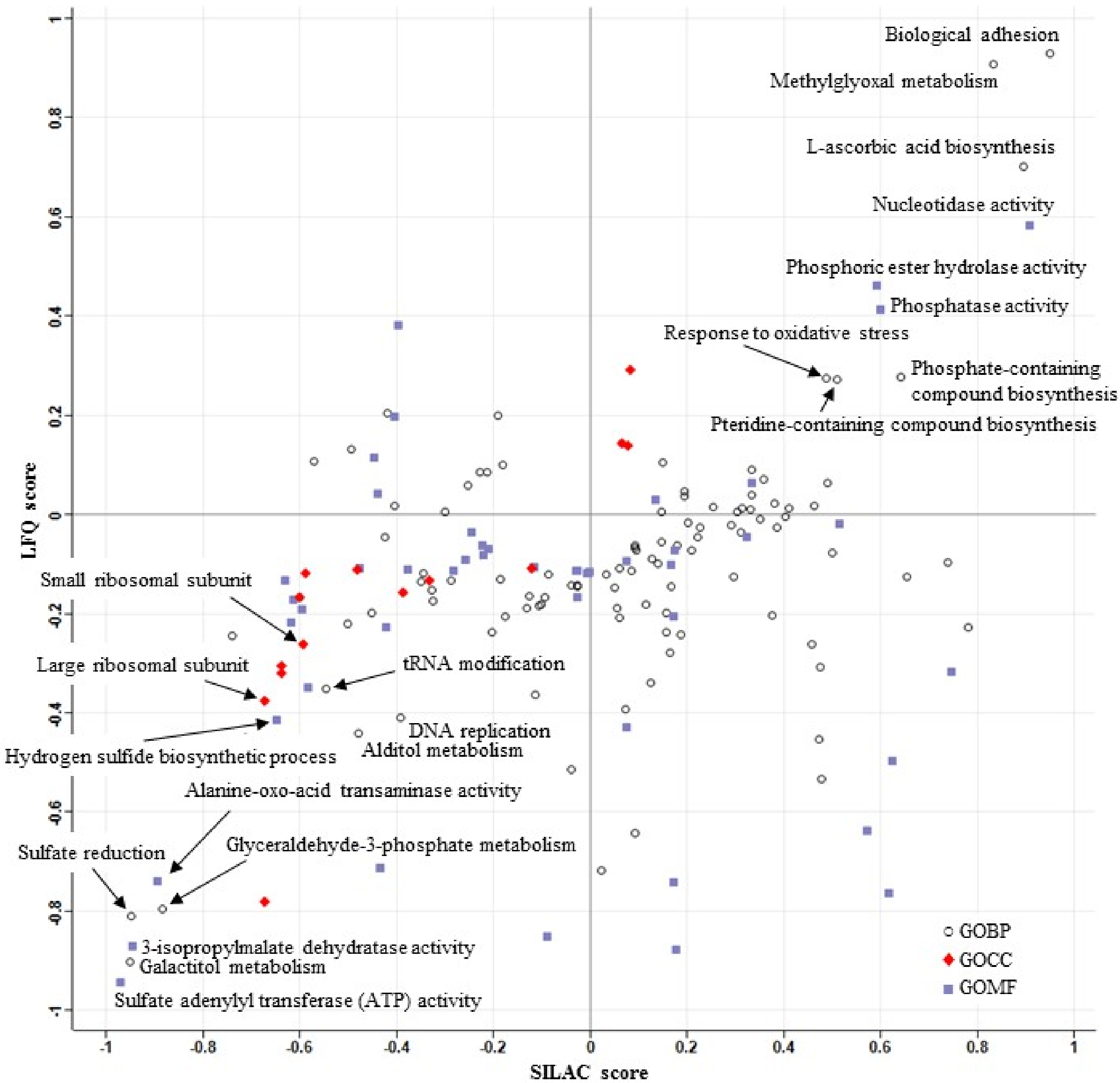

Figure 2. Two-dimensional GO term annotation enrichment of SILAC- *(x)* and LFQ-quantified *(y)* proteins. *Scatter plot*, GO terms plotted against *x* and *y* according to their correlation *s*-score. *GOBP*, Gene Ontology biological process category. *GOCC*, Gene Ontology cellular compartment category. *GOMF*, Gene Ontology molecular function category.

Of 822 protein groups analyzed, 44 and 36 displayed more than two-fold up- and downregulation respectively (Fig.1, Table S6). In order to narrow down the input data for GO term enrichment to entries that actually display the differential expression, we used the lists of the mentioned 44 and 36 proteins as queries for DAVID Bioinformatic Resources 6.7 online database-based annotation enrichment tool with subsequent assignment of the proteins to metabolic pathways prospectively affected by starvation (Table S7). Expectedly, the set of overexpressed proteins displays enrichment of various stress GOBP terms, such as *GO:0050896~response to stimulus*, *GO:0006950~response to stress*, *GO:0009597~detection of virus*, etc. Stress-related pathways, as discussed below, tend to implicate the same key players and to a large extent entail overlapping physiological and morphological changes, thus explaining the involvement of seemingly irrelevant terms, such as *'Detection of virus'*, in a starvation response.

DISCUSSION

For most microbes in their natural habitats starvation is a norm with a state of satiety being a seldom event (Kolter, Siegele & Tormo, 1993; Finkel, 2006). In accordance with this logic, the reports describing microbial features in nurtrient-enriched laboratorial environments do not fully convey the nuances of an inner cellular dynamics in regard to the cell's natural milieu. Given the implication of starvation, on par with other frequent naturally occurring stresses, in modulation of pathogenicity, the study of '-omic' changes secondary to prolonged periods of nutrients deficiency presents a task of valid medical importance. Remarkably, few publications, to our knowledge, deal with this topic with the breadth of a shotgun proteomics with one notable exception being the paper by Soares et al. (Soares et al., 2013). However, despite the comprehensiveness of their study, the authors do not look into the starvation per se instead interrogating the cells at a late stationary phase which we believe is qualitatively different from the complete absence of any external nutrients, and surely, as mentioned above, is far from what bacteria face in nature. In order to fill the described gap, we combined label-free and SILAC approaches to perform comparative quantitative proteomic analysis of log-phase and carbon starved bacteria using high-throughput mass spectrometric pipeline.

It is known, that nutritional downshift results in a rapid expression of a number of survival-promoting stress modulators, most notably an integration host factor (IHF) (Nyström, 1995), an alternative RNA polymerase sigma factor rpoS (Dong & Schellhorn, 2009; Sharma & Chatterji, 2010), and a guanosine tetraphosphate (ppGpp) (Traxler et al., 2008). Bibliographical survey of the proteins, found to be upregulated by starvation, expectedly revealed the involvement of some of them with the various stress networks controlled by said modulators. For instance, it had been shown that universal stress proteins, represented in our list of upregulated proteins by E and F family members, are controlled by ppGpp and confer resistance to oxidative agents (Nachin, Nannmark & Nyström, 2005), and UV-induced DNA damage (Diez, Gustavsson & Nyström, 2000; Gustavsson, Diez & Nyström, 2002). Osmotically-inducible protein Y, downstream rpoS, is upregulated at stationary phase and protects against hyperosmolarity (Yim & Villarejo, 1992; Dong & Schellhorn, 2009).

One of the most prominent hallmarks of starvation in bacteria is so-called 'stringent response' – a state, regulated by ppGpp, of severe diminution of de novo protein synthesis and intensified turnover of pre-existing proteins (Poltrykus & Cashel, 2008; Kuroda, 2006). Moreover, it had been reported that for survival under starvation cells equally require both unhampered protein degradation (Reeve, Bockman & Matin, 1984), and synthesis (Reeve, Amy & Matin, 1984). The observed downregulation of ribosomal proteins may in part be explained by their elimination by the Lon protease which had been shown to break the former down in response to the accumulation of ppGpp (Kuroda, 2006). Upregulated ribosome-associated inhibitor A (RaiA) further contributes to attenuation of protein synthesis by binding to ribosomal A-site and impeding polypeptide chain elongation (Agafonov & Spirin, 2004). YqjD, with paralogous ElaB and YgaM, binds to 70S and 100S ribosomes and is implicated in translation inhibition (Yoshida et al., 2012).

One might note that 50S ribosomal subunit protein L31 is actually upregulated upon starvation rendering the link between the nutrient insufficiency and downregulation of ribosomal proteins dubious. However, in *Bacillus subtilis* the L31 protein exists in two paralogs, RpmE and YtiA (Gabriel & Helmann, 2009; Nanamiya & Kawamura, 2010), whose incorporation into a ribosome in dependent on intracellular zinc concentration. Under zinc-limiting conditions the expression of zinc-binding motif-lacking YtiA is induced by derepression of its gene by transcriptional repressor Zur (zinc uptake regulator) consequently displacing the zinc-binding motif-containing RpmE from a ribosome. Upregulation of zinc uptake proteins ZnuA and ZinT could serve as an indication of undercurrent zinc deficiency (Bhubhanil et al., 2014) but, taking into consideration the equal presence of Zur in starved and exponentially growing cells, one may assume that the deficiency is not pronounced enough to elicit a universal exchange of RpmE for YtiA.

Upon entry to stationary phase bacterial cells undergo dramatic morphological changes. They are smaller in size (Grossman, Ron & Woldringh, 1982) which is in accord with overall downregulation of protein synthesis described in the previous paragraphs. Stationary-phase *Escherichia coli* cells develop increased cell envelope resilience and pressure resistance as well (Charoenwong, Andrews & Mackey, 2011) reflecting the changes in cell wall structure aimed at withstanding challenges of stress.

Starved bacterial cells display tendency towards grouping: the failure to separate after division, described in (Wainwright et al., 1999), results in filamentous growth which conforms to the reports of UspE-dependent cell aggregation (Nachin, Nannmark & Nyström, 2005), and transcriptional factor BolA-mediated biofilm formation (Guinote et al., 2014; Dressaire et al., 2015) (both of the proteins were upregulated by starvation). Our 2D annotation analysis unequivocally showed that starved cells are enriched in *'Biological adhesion'* GO term and thus underscores the importance of said pathway in survival under stress.

Formation of cellular aggregates, such as biofilms, could serve as a defense mechanism against antibiotics as described in (Nguyen et al., 2011; Bernier et al., 2013). However, it seems that starved cells also possess protection against general broad-spectrum biocides: e.g., it had been reported that energy substrate limitation might diminish the microbial susceptibility to benzalkonium (Luppens, Abee & Oosterom, 2001; Bjergbaek et al., 2008), as well as to common disinfectants such as chlorine (Lisle et al., 1998; Saby, Leroy & Block, 1999). Moreover, starved *Streptococcus mutans* exhibited higher resistance to anti-cancer agents NaF and chlorhexidine acetate (Tong et al., 2011). Thus, given the readily involvement of adhesion mechanisms in response to stress, it is extremely important to understand the peculiarities of cell aggregation in relation to antibiotic resistance.

As we have shown, stress-related pathways form an intricate web of connections often recruiting the same key players to respond to different stimuli. The elucidation and verification of the role of each player and its possible companions would require a separate study. However, the insights gained by the large-scale proteomics can outline possible 'angles of attack' to address some problems. For example, the iron transporter FecA, shown in our study to be overexpressed in response to the absence of nutrients, had been shown to be essential for superoxide dismutase (SOD) activation in *Helicobacter pylori* (Tsugawa et al., 2012), whereas in *Shigella flexneri* it is associated with pathogenicity and antibiotic resistance (Luck et al., 2001). Given the importance of iron transporters in cell survival under oxidative stress (Nicolaou et al., 2013), the verification of implication of FecA in *Escherichia coli* stress endurance might prove promising.

In our study we were especially interested in uncharacterized entries on our list of proteins accumulated during starvation. Although with unknown function, some of them have already been identified as participants in various stress-regulated networks: YgaU and YahO had been identified as members of rpoS regulon (Ibanez-Ruiz et al., 2000; Lacour & Landini, 2004) with former partaking in osmotic stress response (Weber, Kögl & Jung, 2006), and cell wall remodeling (Bernal-Cabas, Ayala & Raivio, 2015); YqjD, as mentioned earlier, is an inner membrane protein associated with stationary-phase ribosomes (Yoshida et al., 2012); a putative lipoprotein YbjP, regulated by rpoS (Lacour & Landini, 2004), and YahK partake in biofilm formation (Tenorio et al., 2003; May & Okabe, 2011). However, for YdcL, YbeL, YibT, YnfD, YccU, YicH and YpfJ the present report is the first to ascribe them to a stress-induced proteome. Interestingly, we noticed some discrepancy between our results and the ones gained by Soares et al. (Soares et al., 2013) in regard to the uncharacterized proteins. In particular, in their study the authors could not detect YdeL, YgaM and YnfD at any stage, whereas for YbeL they report steady decrease in abundance as cells proceed through growth phases. For YibT they observed a sharp two-fold upregulation of the protein at an early stationary phase (T4) with subsequent three-fold decline at a late stationary phase (T5). YpfJ followed the inverse pattern displaying the lowest abundance at T4 with signs of reversion at T5. YdcL, YicH and YahK did not display any significant change in abundance throughout the time range.

As we have discussed earlier, an acute energy source withdrawal that leads to abrupt starvation-induced stress response, and nutrient-depleted late stationary phase differ in nature and, therefore, may theoretically affect different regulatory nodes and/or implicate them to greater or lesser degree. It is entirely possible that the discrepancy between the data described in the previous paragraph stems from the differences in approaches to starvation, although the choice of the working strain and sample preparation routine must too be taken into consideration.

CONCLUSIONS

To summarize, we performed a broad study of the *Escherichia coli* proteome afflicted by carbon starvation. We identified 44 proteins implicated in a number of stress resistance networks whose expression was positively affected by the absence of nutrients. The bibliographical search corroborated our findings since many of the proteins had been shown to be regulated by various known stress modulators. However, the data gathered contains a vast body of useful information on thousands of proteins and peptides not represented in the present report. Our data is publicly available for possible inquiries through the ProteomeXchange Consortium repository.

REFERENCES

Agafonov DE., Spirin AS. 2004. The ribosome-associated inhibitor A reduces translation errors. *Biochemical and biophysical research communications* 320:354–8.

Ashburner M., Ball CA., Blake JA., Botstein D., Butler H., Cherry JM., Davis AP., Dolinski K., Dwight SS., Eppig JT., Harris MA., Hill DP., Issel-Tarver L., Kasarskis A., Lewis S., Matese JC., Richardson JE., Ringwald M., Rubin GM., Sherlock G. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature genetics* 25:25–9.

Bernal-Cabas M., Ayala JA., Raivio TL. 2015. The Cpx envelope stress response modifies peptidoglycan cross-linking via the L,D-transpeptidase LdtD and the novel protein YgaU. *Journal of bacteriology* 197:603–14.

Bernier SP., Lebeaux D., DeFrancesco AS., Valomon A., Soubigou G., Coppée J-Y., Ghigo J-M., Beloin C. 2013. Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. *PLoS genetics* 9:e1003144.

Bhubhanil S., Sittipo P., Chaoprasid P., Nookabkaew S., Sukchawalit R., Mongkolsuk S. 2014. Control of zinc homeostasis in *Agrobacterium tumefaciens* via zur and the zinc uptake genes znuABC and zinT. *Microbiology (Reading, England)* 160:2452–63.

Bjergbaek LA., Haagensen JAJ., Molin S., Roslev P. 2008. Effect of oxygen limitation and starvation on the benzalkonium chloride susceptibility of *Escherichia coli*. *Journal of applied microbiology* 105:1310–7.

Bokinsky G., Baidoo EEK., Akella S., Burd H., Weaver D., Alonso-Gutierrez J., García-Martín

H., Lee TS., Keasling JD. 2013. HipA-triggered growth arrest and β-lactam tolerance in *Escherichia coli* are mediated by RelA-dependent ppGpp synthesis. *Journal of bacteriology* 195:3173–82.

Brosch M., Yu L., Hubbard T., Choudhary J. 2009. Accurate and sensitive peptide identification with Mascot Percolator. *Journal of proteome research* 8:3176–81.

Charoenwong D., Andrews S., Mackey B. 2011. Role of rpoS in the development of cell envelope resilience and pressure resistance in stationary-phase *Escherichia coli*. *Applied and environmental microbiology* 77:5220–9.

Cox J., Mann M. 2012. 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. *BMC bioinformatics* 13 Suppl 1:S12.

Diez A., Gustavsson N., Nyström T. 2000. The universal stress protein A of *Escherichia coli* is required for resistance to DNA damaging agents and is regulated by a RecA/FtsK-dependent regulatory pathway. *Molecular microbiology* 36:1494–503.

Dong T., Schellhorn HE. 2009. Global effect of RpoS on gene expression in pathogenic *Escherichia coli* O157:H7 strain EDL933. *BMC genomics* 10:349.

Dressaire C., Moreira RN., Barahona S., Alves de Matos AP., Arraiano CM. 2015. BolA is a transcriptional switch that turns off motility and turns on biofilm development. *mBio* 6:e02352–14.

Fang FC., Libby SJ., Buchmeier NA., Loewen PC., Switala J., Harwood J., Guiney DG. 1992. The alternative sigma factor katF (rpoS) regulates *Salmonella virulence*. *Proceedings of the National Academy of Sciences of the United States of America* 89:11978–82.

Finkel SE. 2006. Long-term survival during stationary phase: evolution and the GASP phenotype. *Nature reviews. Microbiology* 4:113–20.

Gabriel SE., Helmann JD. 2009. Contributions of Zur-controlled ribosomal proteins to growth under zinc starvation conditions. *Journal of bacteriology* 191:6116–22.

Grossman N., Ron EZ., Woldringh CL. 1982. Changes in cell dimensions during amino acid starvation of *Escherichia coli*. *Journal of bacteriology* 152:35–41.

Guinote IB., Moreira RN., Barahona S., Freire P., Vicente M., Arraiano CM. 2014. Breaking through the stress barrier: the role of BolA in Gram-negative survival. *World journal of microbiology & biotechnology* 30:2559–66.

Gustavsson N., Diez A., Nyström T. 2002. The universal stress protein paralogues of *Escherichia coli* are co-ordinately regulated and co-operate in the defence against DNA damage. *Molecular microbiology* 43:107–17.

Huang DW., Sherman BT., Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* 4:44–57.

Ibanez-Ruiz M., Robbe-Saule V., Hermant D., Labrude S., Norel F. 2000. Identification of RpoS (sigma(S))-regulated genes in *Salmonella enterica* serovar typhimurium. *Journal of bacteriology* 182:5749–56.

Kolter R., Siegele DA., Tormo A. 1993. The stationary phase of the bacterial life cycle. *Annual review of microbiology* 47:855–74.

Kuroda A. 2006. A polyphosphate-lon protease complex in the adaptation of *Escherichia coli* to amino acid starvation. *Bioscience, biotechnology, and biochemistry* 70:325–31.

Lacour S., Landini P. 2004. SigmaS-dependent gene expression at the onset of stationary phase in Escherichia coli: function of sigmaS-dependent genes and identification of their promoter sequences. *Journal of bacteriology* 186:7186–95.

Lisle JT., Broadaway SC., Prescott AM., Pyle BH., Fricker C., McFeters GA. 1998. Effects of starvation on physiological activity and chlorine disinfection resistance in *Escherichia coli* O157:H7. *Applied and environmental microbiology* 64:4658–62.

Luck SN., Turner SA., Rajakumar K., Sakellaris H., Adler B. 2001. Ferric dicitrate transport system (Fec) of *Shigella flexneri* 2a YSH6000 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. *Infection and immunity* 69:6012–21.

Luppens SB., Abee T., Oosterom J. 2001. Effect of benzalkonium chloride on viability and energy metabolism in exponential- and stationary-growth-phase cells of *Listeria monocytogenes*. *Journal of food protection* 64:476–82.

Mann M. 2014. Fifteen years of stable isotope labeling by amino acids in cell culture (SILAC). *Methods in molecular biology (Clifton, N.J.)* 1188:1–7.

May T., Okabe S. 2011. Enterobactin is required for biofilm development in reduced-genome *Escherichia coli*. *Environmental microbiology* 13:3149–62.

Megger DA., Bracht T., Meyer HE., Sitek B. 2013. Label-free quantification in clinical proteomics. *Biochimica et biophysica acta* 1834:1581–90.

Nachin L., Nannmark U., Nyström T. 2005. Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and motility. *Journal of bacteriology* 187:6265–72.

Nanamiya H., Kawamura F. 2010. Towards an elucidation of the roles of the ribosome during different growth phases in *Bacillus subtilis*. *Bioscience, biotechnology, and biochemistry* 74:451–61.

Nguyen D., Joshi-Datar A., Lepine F., Bauerle E., Olakanmi O., Beer K., McKay G., Siehnel R., Schafhauser J., Wang Y., Britigan BE., Singh PK. 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science (New York, N.Y.)* 334:982–6.

Nicolaou SA., Fast AG., Nakamaru-Ogiso E., Papoutsakis ET. 2013. Overexpression of fetA (ybbL) and fetB (ybbM), encoding an iron exporter, enhances resistance to oxidative stress in *Escherichia coli*. *Applied and environmental microbiology* 79:7210–9.

Nyström T. 1995. Glucose starvation stimulon of *Escherichia coli*: role of integration host factor in starvation survival and growth phase-dependent protein synthesis. *Journal of bacteriology*

177:5707–10.

Petrosino JF., Galhardo RS., Morales LD., Rosenberg SM. 2009. Stress-induced β-lactam antibiotic resistance mutation and sequences of stationary-phase mutations in the *Escherichia coli* chromosome. *Journal of Bacteriology* 191:5881–5889.

Potrykus K., Cashel M. 2008. (p)ppGpp: still magical? *Annu Rev Microbiol.* 62:35-51.

Poole K. 2012. Bacterial stress responses as determinants of antimicrobial resistance. *The Journal of antimicrobial chemotherapy* 67:2069–89.

Prax M., Bertram R. 2014. Metabolic aspects of bacterial persisters. *Frontiers in Cellular and Infection Microbiology* 4:148.

Reeve CA., Amy PS., Matin A. 1984. Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *Journal of bacteriology* 160:1041–6.

Reeve CA., Bockman AT., Matin A. 1984. Role of protein degradation in the survival of carbon-starved *Escherichia coli* and *Salmonella typhimurium*. *Journal of bacteriology* 157:758–63.

Saby S., Leroy P., Block JC. 1999. *Escherichia coli* resistance to chlorine and glutathione synthesis in response to oxygenation and starvation. *Applied and environmental microbiology* 65:5600–3.

Sharma UK., Chatterji D. 2010. Transcriptional switching in *Escherichia coli* during stress and starvation by modulation of sigma activity. *FEMS microbiology reviews* 34:646–57.

Soares NC., Spät P., Krug K., Macek B. 2013. Global dynamics of the *Escherichia coli* proteome and phosphoproteome during growth in minimal medium. *Journal of proteome research* 12:2611–21.

Suh SJ., Silo-Suh L., Woods DE., Hassett DJ., West SE., Ohman DE. 1999. Effect of rpoS mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *Journal of bacteriology* 181:3890–7.

Tenorio E., Saeki T., Fujita K., Kitakawa M., Baba T., Mori H., Isono K. 2003. Systematic characterization of *Escherichia coli* genes/ORFs affecting biofilm formation. *FEMS microbiology letters* 225:107–14.

Thompson LJ., Merrell DS., Neilan BA., Mitchell H., Lee A., Falkow S. 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. *Infection and immunity* 71:2643–55.

Tong Z., Tao R., Jiang W., Li J., Zhou L., Tian Y., Ni L. 2011. In vitro study of the properties of *Streptococcus mutans* in starvation conditions. *Archives of oral biology* 56:1306–11.

Traxler MF., Summers SM., Nguyen H-T., Zacharia VM., Hightower GA., Smith JT., Conway T. 2008. The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. *Molecular microbiology* 68:1128–48.

Tsugawa H., Suzuki H., Matsuzaki J., Hirata K., Hibi T. 2012. FecA1, a bacterial iron transporter, determines the survival of *Helicobacter pylori* in the stomach. *Free radical biology & medicine* 52:1003–10.

Vizcaíno JA., Côté RG., Csordas A., Dianes JA., Fabregat A., Foster JM., Griss J., Alpi E., Birim M., Contell J., O'Kelly G., Schoenegger A., Ovelleiro D., Pérez-Riverol Y., Reisinger F., Ríos D., Wang R., Hermjakob H. 2013. The PRoteomics IDEntifications (PRIDE) database and associated tools: status in 2013. *Nucleic acids research* 41:D1063–9.

Wainwright M., Canham LT., al-Wajeeh K., Reeves CL. 1999. Morphological changes (including filamentation) in *Escherichia coli* grown under starvation conditions on silicon wafers and other surfaces. *Letters in applied microbiology* 29:224–7.

Weber A., Kögl SA., Jung K. 2006. Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli*. *Journal of bacteriology* 188:7165–75.

Wiśniewski JR., Zougman A., Nagaraj N., Mann M. 2009. Universal sample preparation method for proteome analysis. *Nature methods* 6:359–62.

Yim HH., Villarejo M. 1992. osmY, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. *Journal of bacteriology* 174:3637–44.

Yoshida H., Maki Y., Furuike S., Sakai A., Ueta M., Wada A. 2012. YqjD is an inner membrane protein associated with stationary-phase ribosomes in *Escherichia coli*. *Journal of bacteriology* 194:4178–83.