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Dynamics of the *Escherichia coli* proteome in response to nitrogen starvation and entry into the stationary phase

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ABSTRACT

Nitrogen is needed for the biosynthesis of biomolecules including proteins and nucleic acids. In the absence of fixed nitrogen prokaryotes such as *E. coli* immediately ceases growth. Ammonium is the preferred nitrogen source for *E. coli* supporting the fastest growth rates. Under conditions of ammonium limitation, *E. coli* can use alternative nitrogen sources to supply ammonium ions and this reprogramming is led by the induction of the NtrC regulon. Here we used label free proteomics to determine the dynamics of *E. coli* proteins expression in response to ammonium starvation in both the short (30 min) and the longer (60 min) starvation. Protein abundances and post-translational modifications confirmed that activation of the NtrC regulon acts as the first line of defense against nitrogen starvation. The ribosome inactivating protein Rmf was induced shortly after ammonium exhaustion and this was preceded by induction of other ribosome inactivating proteins such as Hpf and RaiA supporting the hypothesis that ribosome shut-down is a key process during nitrogen limitation stress. The proteomic data revealed that growth arrest due to nitrogen starvation correlates with the accumulation of proteins involved in DNA condensation, RNA and protein catabolism and ribosome hibernation. Collectively, these proteome adaptations will result in metabolic inactive cells which are likely to exhibit multidrug tolerance.

1. Introduction

Prokaryotes have to face a myriad of nutritional conditions throughout their life cycles; deprivation of one or more nutrients represents a challenge for reproduction and survival. Not surprisingly, microbes carry a plethora of signaling and adaptation systems to cope with limitation of nutrients. Nitrogen (N) is a key nutrient required for protein and nucleic acid synthesis. Bacteria can utilize a vast range of compounds as nitrogen sources such ammonium ions, nitrogen oxides, amino acids, urea or nitrogen gas (in the case of diazotrophs). For most prokaryotes, including *Escherichia coli*, ammonium ions is the preferred N-source as it promotes shorter doubling times and represses the use of other nitrogen sources [1].

In *E. coli*, ammonium is mainly assimilated through the glutamine synthetase (GS), glutamate dehydrogenase (GOGAT) pathway. GS incorporates inorganic ammonium into glutamate producing glutamine, while GOGAT uses glutamine (produced by GS) and 2-oxoglutarate to produce two molecules of glutamate. Glutamine and glutamate act as main N-donors for other biosynthetic reactions [2]. The N-status in *E. coli* is reflected by the intracellular levels of glutamine and 2-oxoglutarate, such that when ammonium is limiting, glutamine is low and 2-oxoglutarate is high [2–4].

The best-characterized N-sensor modules in *E. coli* are the two P_{II} proteins, GlnB and GlnK. Under low or limiting glutamine concentrations GlnD catalyzes P_{II} uridylylation. Conversely, under high glutamine, GlnD removes uridylyl groups from P_{II}-UMP [5]. The P_{II} proteins directly senses the 2-oxoglutarate levels through allosteric binding [6,7]. The structural status of the P_{II} proteins directly affects the activity of four protein targets in *E. coli*; 1) the ammonium transporter AmtB [8]; 2) the acetyl-CoA carboxylase enzyme [9,10]; 3) the ATase enzyme, responsible for regulating GS activity through reversible adenylylation [11]; and 4) the NtrB sensor protein, belonging to the NtrBC two-component transcriptional regulatory system [12].

Under ammonium limitation, GlnB-UMP accumulates favoring phosphorylation of NtrC by NtrB [12]. NtrC-P activates transcription of a variety genes involved in the use of alternative nitrogen sources, thus initiating the N-scavenging response [13]. Conversely, when

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ammonium is abundant, unmodified GlnB interacts with NtrB which acts as a NtrC-P phosphatase turning down transcription from sigma 54 (σ^{54}) NtrC-P dependent promoters [14].

Another important adaptive response to N limitation is the activation of the stringent response. When amino acids are limiting, binding of uncharged tRNAs to the A-site at the ribosomes promotes the activation of the RelA enzyme leading to the production of the alarmone (p)ppGpp [15]. This metabolite directly interacts with RNA polymerase reprogramming transcription, so that genes important for survival during starvation are favoured at the expense of those required for growth and proliferation [16]. Recent data showed that the NtrC system is connected to the stringent response as NtrC-P activates *relA* gene transcription under nitrogen starvation [17]. A global proteomic analysis could help to identify novel connections between the classical nitrogen starvation response (i.e. the Ntr system) with other metabolic pathways. Here we used a label-free proteomics approach to describe for the first time the temporal dynamics of the *E. coli* proteome in response to N-starvation.

2. Experimental section

2.1. Cell culture

Fresh colonies of *E. coli* MG1655 strain (wild-type K12 *ilvG*$^+$), kindly provided by Prof. Yeong-Jae Seok, Seoul National University, Korea [18], were cultured in LB medium for 10 h at 120 rpm 37 °C. This culture was used to inoculate at 1:50 ratio M9 media supplemented with 30 mM NH_4Cl as N-source. After 16 h at 120 rpm 37 °C, this culture was used to inoculate 50 mL of fresh M9 medium containing either 3 or 30 mM NH_4Cl as nitrogen source to an initial $OD_{600\ nm} = 0.05$. The cultures were maintained at 120 rpm 37 °C with the $OD_{600\ nm}$ monitored every 30 min.

Aliquots of the cultures were collected on ice at the time point indicated in each experiment and were subjected to centrifugation at 3000*g*, 4 °C for 15 min. Cells were suspended in 50 mM Tris-HCl pH 7.5 containing 100 mM KCl and disrupted by sonication on ice. The soluble fraction was collected by centrifugation at 20,000*g*, 4 °C for 15 min and the protein content determined using the Bradford assay (Sigma). Aliquots containing 100 μg of protein were frozen at −80 °C, lyophilized and sent as dried powder to the Proteomics Core Facility, Rikshospitalet, Oslo, Norway for LC/MS/MS analysis.

Three independent cultures were used for each condition, generating three independent protein samples that were subjected to three independent LC/MS/MS runs (see below).

2.2. Label free quantitative LC-MS/MS analysis

For LC-MS/MS analysis 15 μg of each sample were independently suspended in 50 μL of ammonium bicarbonate 100 mM pH 8.0 and reduced using 1 μL of DTT 1 mM for 45 min. Proteins were denatured by heating at 95 °C for 30 min, modified trypsin (Promega) was added to a 1:50 ratio and incubated overnight at 37 °C in a wet chamber. Protein digestion was quenched with TFA 3% (v/v). The sample was cleaned using C18 STAGE-TIPs as described [19] and resuspended in 10 μL of 0.1% formic acid. Each protein sample was run in 3 μL injections. Peptide separation was performed on a nano-HPLC (EASY nLC1000, Thermo) using a reverse phase column of 25 cm, 75 μm of internal diameter and 2 μm particles. Peptides were eluted in 120 min runs using a linear gradient of 2 to 30% of organic solvent (100% acetonitrile and 0.1% formic acid) at 300 $nL \cdot min^{-1}$.

Samples were analyzed in a QExactive Orbitrap (Thermo Scientific). Sample acquisition parameters for MS scans were: microscans 1; resolution 70,000 at m/z 200; AGC target 3e6; maximum injection time 20 ms; scan range: 400–1200 m/z. The parameters for data dependent MS/MS acquisition were: microscans 1; resolution 17,500; AGC target 1e5; maximum injection time 100 ms; loop count 10; isolation width 2.0 m/z; collision energy 25.0%; single charged ions were excluded; and dynamic exclusion of 30 s.

QExactive Orbitrap data was processed using MaxQuant v1.5.2.8 [20], using the following parameters: variable modifications methionine oxidation; N-acetylation of N-terminal; and conversion of glutamine and glutamate to pyro-glutamate. The first search was performed using 20 ppm error and the main search 6 ppm; maximum of two missed cleavages were allowed. Protein and peptide FDR threshold of 0.01; Min Unique Peptides: 1; Min Peptide Length: 7; second peptides option ON; match between runs ON, with Time Window of 2 min; label-free quantitation ON, with minimal ratio count 2; iBAQ ON with log fit ON. Proteins were identified using an Uniprot *E. coli* K12 database from June 2014 (4315 entries). Additional searches including tyrosine uridylylation and adenylylation as variable modifications were performed.

Statistical analysis was performed using MaxQuant – Perseus package version 1.5.0.30. Briefly, output with identified proteins from MaxQuant (proteingroups.txt) was loaded and normalized area under curve values were assigned for each protein. Proteins identified from contaminants and reversed sequences (from FDR calculations) were removed. Quantitative values were logarithmized at base 10, and non-assigned values (i.e., quantitation equal zero before log calculation) were replaced by normal distribution of the data (Width 0.3, Down shift 1.8). Statistically significant differences were assigned using two-way *t*-test with *p* value threshold of 0.05 and permutation-based FDR correction allowing 250 permutations.

2.3. Expression of promoter:gfp fusions

The expression of selected genes was monitored by flow cytometry using a plasmid-born library of *E. coli* promoters transcriptional fused to *gfp* (GE-Healthcare) [21]. *E. coli* MG1655 carrying the selected gene fusions were cultivated in triplicate exactly as described for proteins extraction (except for the presence of kanamycin 100 μg/mL used for plasmid maintenance). Cell growth was monitored by determining the $OD_{600\ nm}$. Cells cultured under low nitrogen regime (−N, 3 mM NH_4^+) were collected at two different time points after ammonium run out and growth arrest: 30 min (−N T1) and 60 min (−N T2). Cells cultured under high ammonium regime (+N, 30 mM NH_4^+) were collected at similar $OD_{600\ nm}$ as −N T1 and T2, 300 μL aliquots were collected and centrifuged for 1 min at 13000*g*. The supernatant was discarded and the cell pellet resuspended in 1 mL of TBAC (PBS buffer containing 1 mM EDTA and 0.01% v/v Tween 20). The samples were kept on ice and analyzed within 3 h in a flow cytometer BD Accuri C5® equipped with 488 nm laser excitation. To monitor GFP fluorescence 100,000 events were acquired for each sample using the FL1-H (510/15 nm) channel. Cytometer calibration was performed using 8-peack beads (BD Accuri™) according manufacture instruction. The data was expressed as % of the average fluorescence in +N condition ± SD. Statistical analysis was performed using unpaired *t*-test in GraphPad Prism 6.0 $^*p < 0.05$.

3. Results

In order to determine how *E. coli* adapts its proteome to cope with a reduction in the availability of ammonium, cells were cultured in minimum media containing high nitrogen N+ (30 mM NH_4Cl) or low nitrogen N− (3 mM NH_4Cl). Under N− conditions, cells grew exponentially until an $OD_{600\ nm}$ of ~0.6 was reached, at this point all ammonium is consumed by the cells (the −N condition can be considered as N-run out condition) resulting in growth arrest [17]. Cells were collected at two time points after growth arrest (30 min T1 and 60 min T2, Fig. 1A). Cells cultivated under ammonium abundance had no growth restriction and were collected at the middle of exponential phase at a similar $OD_{600\ nm}$ ~0.6 (Fig. 1B).