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# Proteome analysis of an *Escherichia coli ptsN*-null strain under different nitrogen regimes

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ABSTRACT

The carbohydrate-uptake phosphorelay PTS system plays a key role in metabolic regulation in Bacteria controlling the utilization of secondary carbon sources. Some bacteria, such as *Escherichia coli*, encode a paralogous system named PTS$^{Ntr}$ (nitrogen related PTS). PTS$^{Ntr}$ is composed of EI$^{Ntr}$ (*ptsP*), NPr (*ptsO*), and EIIA$^{Ntr}$ (*ptsN*). These proteins act as a phosphorelay system from phosphoenolpyruvate to EI$^{Ntr}$, NPr and them to EIIA$^{Ntr}$. PTS$^{Ntr}$ is not involved in carbohydrate uptake and it may be dedicated to performing regulatory functions. The phosphorylation state of EI$^{Ntr}$ is regulated by allosteric binding of glutamine and 2-oxoglutarate, metabolites whose intracellular levels reflect the nitrogen status. Although PTS$^{Ntr}$ is designated as having nitrogen-sensory properties, no major effect of this system on nitrogen regulation has been described in *E. coli*. Here we show that an *E. coli ptsN* deletion mutant has impaired growth in minimal medium. Proteome analysis of the Δ*ptsN* strain under different nitrogen regimes revealed no involvement in regulation of the canonical nitrogen regulatory (Ntr) system. The proteomic data support the conclusion that *ptsN* is required to balance the activities of the sigma factors RpoS and RpoD in such way that, in the absence of *ptsN*, RpoS-dependent genes are preferentially expressed.

*Significance:* The nitrogen related PTS$^{Ntr}$ phosphorelay system has been hypothesized to participate in the control of nitrogen metabolism. Here we used a proteomics approach to show that an *Escherichia coli ptsN* null strain, which misses the final module of PTS$^{Ntr}$ phosphorelay, has no significant effects on nitrogen metabolism under different nitrogen regimes. We noted that *ptsN* is required for fitness under minimal medium and for the proper balance between RpoS and sigma 70 activities in such way that, in the absence of *ptsN*, RpoS-dependent genes are preferentially expressed.

## 1. Introduction

The regulation of metabolism is vital for survival, fitness and adaptation of microbial species. Metabolic regulation is particularly important under changing environmental conditions such as nutrient availability. Regulation of bacterial metabolism is complex and multifaceted. Regulatory circuits range from adaptations occurring at longer time scales, such as transcription reprogramming, to rapid ones such as allosteric binding of signaling metabolites and protein post-translational modifications [1].

The *Escherichia coli* PTS (Phospho Transfer System) is one example of a metabolic regulatory module acting at multiple time scales. The PTS is a phosphorylation cascade that couples the transport of sugars to its phosphorylation [2]. The EI protein catalyzes its autophosphorylation using phosphoenolpyruvate as phosphoryl donor; the phosphoryl group is transferred to Hpr, then to the membrane-associated EII component, being finally transferred to the incoming glucose [3]. The availability of glucose affects the phosphorylation status of the PTS proteins; this information is transduced to multiple protein targets that interact with the PTS components in a phosphorylation-dependent manner. One example is the activation of adenylate cyclase under low glucose due to accumulation of phosphorylated EIIA [3,4].

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In addition to the sugar-related PTS, some Bacteria encode a paralogous system named $PTS^{Ntr}$ (*nitrogen related* PTS). $PTS^{Ntr}$ is composed of $EI^{Ntr}$ (*ptsP*), NPr (*ptsO*), and $EIIA^{Ntr}$ (*ptsN*). These proteins act as a phosphorelay system from phosphoenolpyruvate then to the $EI^{Ntr}$, NPr and $EIIA^{Ntr}$ [5–7]. $PTS^{Ntr}$ is not involved in carbohydrate uptake and it may be dedicated to perform regulatory functions. This hypothesis is supported by the presence of a GAF domain in $EI^{Ntr}$, a domain involved in binding of small molecules to sensor proteins [7]. Despite the designation *nitrogen related* PTS, no major effect of this system on nitrogen regulation has been described in *E. coli* to date. In fact, the naming of this system has been a subject of debate [8].

The best described nitrogen regulatory module in *E. coli* and related bacteria is the global nitrogen regulatory system (Ntr) [9]. *E. coli* can use a range of compounds as nitrogen source; however, ammonium is preferred over other nitrogen sources. The Ntr system ensures that the genes involved in the use of alternative nitrogen sources are only expressed when ammonium runs out [9]. The Ntr system comprises an intricate regulatory network which is centered in the action of the PII proteins, GlnB and GlnK [10]. When ammonium is limiting there is a decrease in intracellular glutamine and the bifunctional GlnD enzyme catalyzes $P_{II}$ uridylylation. When ammonium is abundant, intracellular glutamine increases. Glutamine allosterically binds to GlnD activating the removal of uridylyl groups from $P_{II}$-UMP [11]. In addition to regulation by reversible uridylylation, $P_{II}$ function is also affected by allosteric binding of ATP, ADP and 2-oxoglutarate. The levels of 2-oxoglutarate reflect both the nitrogen and carbon status [12].

The structural status of $P_{II}$ proteins acts as a switch dictating the pace of nitrogen assimilatory pathways. In *E. coli*, $P_{II}$ proteins regulate the activity of the bifunctional ATase enzyme, responsible for the control of glutamine synthetase (GS) activity through reversible adenylylation [13]. Moreover, the $P_{II}$ proteins regulate the phosphorylation status of the transcription factor NtrC [14]. When ammonium is limiting, $P_{II}$-UMP accumulates favoring NtrC phosphorylation by NtrB [14]. NtrC-P activates transcription of sigma-N dependent promoters of genes involved in the use of alternative nitrogen sources [15]. Conversely, when ammonium is abundant, unmodified GlnB interacts with NtrB, which acts as a NtrC-P phosphatase that down regulates transcription from sigma-N, NtrC-P dependent promoters [16].

Glutamine and 2-oxoglurate act as the major metabolic signals reflecting the nitrogen and carbon status to regulate $P_{II}$ protein activity [10,12]. Interestingly, these same metabolites control phosphorylation of $PTS^{Ntr}$ in *E. coli* and related bacteria through allosteric binding to the $EI^{Ntr}$ GAF domain [17–20]. Recent analysis in *Salmonella thyphimurium* showed that $EIIA^{Ntr}$ regulates the activity of a key enzyme in amino sugar biosynthesis, GlmS, through direct protein-protein interaction [20]. Furthermore, $EIIA^{Ntr}$ regulates the accumulation of ppGpp in response to nitrogen starvation by interacting with SpoT in *Caulobacter crescentus* [19]. In *E. coli*, $PTS^{Ntr}$ regulates $K^+$, phosphate and lipopolysaccharide homeostasis [21–24]. However, the participation of $PTS^{Ntr}$ in the regulation of *E. coli* nitrogen metabolism, remains elusive [12]. Here we used a label-free proteomics approach to analyze the effects of a *ptsN* knockout on the proteome of *E. coli* cultivated under different nitrogen regimes.

## 2. Material and methods

### 2.1. Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Supplementary Table S1. As the previous described phenotypes of *E. coli* Δ*ptsN* mutants were traced to the lack of functional *ilvG* allele [25] we used strains derived from genetically reconstituted *ilvG*$^+$ *E. coli* strains MG1655 *ilvG*$^+$ [17] or NCM3722 *ilvG*$^+$ [26] throughout this study. An in-frame deletion of *ptsN* was obtained using P1 phage transduction of the Δ*ptsN::*$Km^r$ allele originated from the Keio collection [27]. The $Km^r$ cassette was removed using the pCP20 plasmid following plasmid removal by growth at 42 °C [28]. The deletion of *ptsN* was confirmed by PCR analysis (Fig. S1) and by DNA sequencing using the primers PtsN-F Ec mutcheck 5′ TAATTGTCCGGGCAATTAGC 3′ and PtsN-R Ec mutcheck 5′ GCGACAGATTTACCTGAACC 3′.

### 2.2. Cell culture

Fresh colonies of the indicated *E. coli* strains were cultured in LB medium for 10 h at 120 rpm 37 °C. This culture was used to inoculate M9 medium supplemented with 30 mM $NH_4Cl$ as nitrogen source. After 16 h at 120 rpm 37 °C, a final culture was prepared by inoculating fresh M9 medium, containing either 3 (− N) or 30 mM (+ N) of $NH_4Cl$ as nitrogen source as indicated in each experiment, to an initial $O.D_{600nm}$ = 0.05. The cultures were maintained at 120 rpm 37 °C and the $O.D_{600nm}$ was recorded. Doubling times were calculated during exponential growth.

For proteomic analysis, 50 ml aliquots of the cultures were collected at $O.D_{600nm}$ of approximately 0.5 (+ N) or 60 min after growth arrest (− N), $O.D_{600nm}$ of approximately 0.5. Cells were subjected to centrifugation at 3000*g*, 4 °C for 15 min, suspended in 50 mM TrisHCl pH 7.5 containing 100 mM KCl and ruptured by sonication on ice. The soluble fraction was collected by centrifugation at 20,000*g* 4 °C for 10 min and the protein content was 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).

### 2.3. Label free quantitative LC-MS/MS analysis

For LC-MS/MS analysis 15 μg of each protein sample were independently suspended in 50 μl of ammonium bicarbonate 100 mM pH 8.0, the sample was reduced with 1 μl of DTT 1 mM for 45 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 [29] and resuspended in 10 μl of 0.1% formic acid. Each protein sample was run in using 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·$min^{-1}$.

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 [30], 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. 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.