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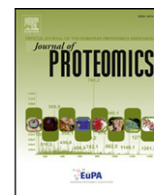

# Global dynamics of *Escherichia coli* phosphoproteome in central carbon metabolism under changing culture conditions

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Little is known about the role of global phosphorylation events in the control of prokaryote metabolism. By performing a detailed analysis of all protein phosphorylation events previously reported in *Escherichia coli*, dynamic changes in protein phosphorylation were elucidated under three different culture conditions. Using scheduled reaction monitoring, the phosphorylation ratios of 82 peptides corresponding to 71 proteins were quantified to establish whether serine (S), threonine (T) and tyrosine (Y) phosphorylation events displayed a dynamic profile under changing culture conditions. The ratio of phosphorylation for 23 enzymes from central carbon metabolism was found to be dynamic. The data presented contributes to our understanding of the global role of phosphorylation in bacterial metabolism and highlight that phosphorylation is an important, yet poorly understood, regulatory mechanism of metabolism control in bacteria.

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## 1. Introduction

Bacterial cells adapt to rapid and drastic environmental changes by turning 'on' and 'off' specific subsets of proteins. Post-translational modifications are the fastest regulatory mechanism by which microorganisms regulate protein activity [1]. Protein phosphorylation, widely distributed in all living organisms, is the most prominent and most studied protein modification. Phosphorylation controls biological functions by inducing conformational changes in the protein's active site or by regulating protein interactions with other proteins [2–7].

In prokaryotes, protein phosphorylation of histidine (H) and aspartate (D) residues occurs commonly as part of two component signalling systems and has been extensively studied [8]. Two component systems rely on high fidelity of recognition between the kinase and the response regulator. Several auxiliary regulators, which interfere with these highly specific phosphotransfer reactions have been reported in the last decade [9]. They act either on the sensory kinases or the response regulators to accomplish signal integration. Accompanying these regulators, increasing evidence shows that phosphorylation of serine (S), threonine (T) and tyrosine (Y) in bacteria, also regulates numerous physiological processes in bacteria, including key aspects of the central carbon metabolism [10–13].

Mass spectrometry based phosphoproteomic studies in *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*) and *Lactococcus lactis* (*L. lactis*) [14–16] confirmed earlier evidence from gel based proteomics [17,18] that S/T/Y phosphorylation is far more widespread in bacteria than suggested by the few well-known examples, e.g., HPr kinase/phosphorylases [19–22] and isocitrate dehydrogenase (Icd) [23–25]. Since then, investigations have flourished; reports have emerged of S/T/Y phosphorylation in *Klebsiella pneumoniae* [26], *Pseudomonas* spp. [27], *Streptomyces coelicolor* [28,29], *Streptococcus pneumoniae* [30], *Mycoplasma pneumoniae* [31], *Listeria monocytogenes* [32], *Helicobacter pylori* [33], *Clostridium acetobutylicum* [34], *Rhodopseudomonas palustris* [35], *Thermus thermophiles* [36], *Synechococcus* sp. [37], *Staphylococcus aureus* [38], *Acinetobacter baumannii* [39], *Saccharopolyspora erythraea* [40], and *Mycobacterium tuberculosis* [41] among others. Conservation across species of phosphorylation sites in enzymes from the central carbon metabolism in pathways for glycolysis, pentose phosphate and the tricarboxylic acid (TCA) cycle, suggests that phosphorylation plays a pivotal role in regulating central carbon metabolism. Previous studies have also linked S/T/Y phosphorylation to regulation of cell differentiation [29], physiology [42,43], stress [44,45], virulence and pathogenicity [46–50].

The majority of bacterial phosphoproteomic studies have focused on phosphor site discovery. However, there is a significant step from the discovery of a phosphor site to assigning biological function and identifying the control mechanisms and relevant phosphokinase and phosphatase. It is often difficult to correctly assign the phosphor site and even when assignment is possible, many discovered sites are likely to be spurious signals due to non-specific kinase activity and phosphortransfer during mass spectrometry [51]. For example, of the 105 phosphor sites originally reported by Macek and colleagues for *E. coli* growing in Luria-Bertani (LB) [15], only nine were found in their subsequent SILAC-based temporal study in glucose minimal M9 medium, while 141 new phosphor sites were reported [12]. It is unclear how many of these differences can be attributed to physiological responses

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(e.g., the absence of the well characterised Icd phosphorylation in presence of glucose) as opposed to spurious signals in either dataset.

For phosphorylation to play a physiological relevant role in metabolic control, however, it must be quantitatively significant and vary under changing culture conditions. In this study, we undertook a detailed quantitative evaluation of all phosphorylation events reported in the first *E. coli* phosphoproteome study grown on three carbon sources. Using scheduled reaction monitoring, we characterised the phosphorylation profile of 82 peptides, of which 71 proteins were differentially phosphorylated, and found that 23 enzymes from the central carbon metabolism displayed a distinct pattern of phosphorylation. These results confirm the process of phosphorylation as a key, yet poorly understood, regulator of *E. coli* metabolism.

## 2. Material and methods

### 2.1. Strain & culture conditions

*E. coli* K-12 MG1655 (CGSC 7740) were initially grown on LB medium. Seed cultures were washed three times with fresh minimal medium (R/2 (supplemented with 20 g/L of glucose) (51) or M9 (supplemented with 2 g/L acetate) (50)) and transferred to shake flasks (2 L cultured at a working volume of 400 mL) cultured in a shaking incubator set to 37 °C. Then, cells were cultured on each medium (LB, glucose, acetate) using a bioreactor (DasGip Control 4.0, Germany). Bioreactor cultures were performed at a working volume of 400 mL, at constant pH 6.8 and a constant airflow of 0.375 v.v.m. Dissolved oxygen tension was controlled above 30% by agitation first and then by mixing air with pure oxygen. Cell growth was monitored by measuring the absorbance at 600 nm (Libra S4, Biochrom Ltd, United Kingdom [UK]).

### 2.2. Protein extractions

Cells harvested in mid-exponential phase were centrifuged at 5250 rpm (Allegra X15R, Beckman Coulter, United States [US]) for 10 min at 4 °C, washed once (washing buffer: 100 mM NaCl, 25 mM Tris–HCl, pH7.5) and resuspended in lysis buffer as described elsewhere with minor modifications [14]. Homogenous cell disruption was achieved by mechanical disruption using 0.1 mm glass beads (5 × 1 min, 4800 rpm). Cellular debris was removed by centrifugation (13,000 rpm for 10 min at 4 °C, microfuge 22R, Beckman coulter, US) and the lysate treated with DNase I (Fermentas) and RNase A (Fermentas). The crude protein extract was extensively dialyzed against MilliQ water containing protease inhibitors using Slide-A-Lyzer cassettes (3500MWCO, Thermo Scientific, US) and protein content was quantified using 2D Quant kit (GE Healthcare, US). Finally, protein extracts were concentrated by lyophilization and stored at −20 °C until protein digestion.

### 2.3. Protein digestion

Ten milligrammes of lyophilized protein were dissolved in denaturing solution as described in [15] supplemented with 2% CHAPS to increase protein solubilization. Samples were reduced with DTT and alkylated with iodoacetamide as described previously [15]. Samples were diluted with 5 volumes of 10 mM ammonium bicarbonate to reduce urea concentration, before being digested with LysC (lysyl endopeptidase) for 3 h. Proteins were further digested overnight with trypsin (Promega Gold, MS grade) at 37 °C with gentle rocking. Prior to down-stream analysis, samples were concentrated by vacuum centrifugation (Eppendorf, Germany) and acidified to pH < 2.7 with 0.1% formic acid. Insoluble materials were removed by centrifugation and the samples dried using a vacuum centrifuge.

### 2.4. Estimation of the lower limit of detection (LLD) for α-casein

To estimate the LLD for *α-casein*, commercial phosphorylated and unphosphorylated *α-casein* (Sigma Aldrich, C8032, C6780) were used. *α-Casein* was digested with trypsin (Promega, sequencing grade) overnight. Subsequently, *α-casein* on its phosphorylated and unphosphorylated form was mixed in cellular lysates or distilled water at various concentrations to determine the lower level of detection of mass spectrometer (Nano-LC QSTAR Elite). To assess the sensitivity of sMRM, samples supplemented with different amounts of unphosphorylated α-casein and a constant amount of phosphorylated *α-casein* were injected in an LC coupled to a QTRAP (Applied Biosystems, US).

### 2.5. Phosphopeptide enrichment

Strong Cation Exchange (SCX) was performed as described elsewhere [14]. Briefly, samples were loaded onto a Resource S column (GE Healthcare, US) in 100% solvent A (5 mM $KH_2PO_4$, 30% acetonitrile, 0.1% trifluoroacetic acid, pH = 2.7) at a flow rate of 1 mL/min. Elution was performed with a gradient of 0–30% solvent B (5 mM $KH_2PO_4$, 30% acetonitrile, 350 mM KCl, 0.1% trifluoroacetic acid, pH 2.7) over 30 min. Seventeen 2-mL fractions were collected, desalted (Sep-Pak tC18, Waters) and concentrated by vacuum centrifugation [52]. The dried samples were acidified with 0.1% formic acid and enriched using Phos-$TiO_2$ kit (GL Sciences Inc., US) according to the manufacturer's protocol.

### 2.6. Protein identification

Protein discovery was performed using a in a Nano-LC QSTAR Elite (AB Sciex, US). Peptides were separated on a $C_{18}$ column (Vydac MS C18 300 Å 150 mm × 0.3 mm, GRACE Davison, Discovery Sciences, USA) operated at 30 °C with a gradient running from 0 to 80% acetonitrile (in 0.1% formic acid) for 105 min at a flow rate of 3 μl/min. Proteins were identified by information-dependent acquisition of the fragmentation spectra of charged peptides with a precursor selection window of 100 to 1800 m/z using enhanced pulsed extraction of fragments. Tandem mass spectra were acquired for 1 s and fragmented peptides were selected for sequencing for 12 s in positive mode. Data were acquired using Analyst software and peptides were identified using the paragon algorithm (Protein Pilot software 4.0, Applied Biosystems, US) [53] and Mascot. All searches were conducted as described by Macek et al. [15], using thorough search, FDR, two missed-cleavages, trypsin, iodoacetamyde and DTT and manual inspection for “good” b and y ions.

### 2.7. Quantitative analysis

Scheduled multiple reaction monitoring (sMRM) was used to quantify phosphorylated peptides using either a QTRAP 5500 or a QTRAP 4000 (Applied Biosystems, US) depending on instrument availability [54,55]. Peptides were separated on an analytical column (100 mm × 2.1 mm 2.6 μm 100 Å Kinetix $C_{18}$, Phenomenex, US) using a 2 to 80% acetonitrile (in MilliQ water with 0.1% formic acid) gradient over 100 min. Eluted peptides were analyzed in positive ion mode. All sMRM coordinates were developed and optimised using MRMPilot (Applied Biosystems, US) as described by the software manufacturer.

### 2.8. Statistical analysis

Initial quantitation was performed in Analyst (1.5.2, Applied Biosystems, US) or MSQuant. Each peak was manually inspected and peptides with unfavourable elution profile (bad resolution), interfering noise or inconsistent elution time across biological and technical repeats were excluded from the analysis. Peak areas were normalized between samples using spiked in α-casein as an external standard and log

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