



# **Escherichia coli glycogen metabolism is controlled by the PhoP-PhoQ regulatory system at submillimolar environmental Mg<sup>2+</sup> concentrations, and is highly interconnected with a wide variety of cellular processes**

Manuel Montero, Gustavo Eydallin, Alejandro M. Viale, Goizeder Almagro, Francisco J. Muñoz, Mehdi Rahimpour, María T. Sesma, Edurne Baroja-Fernández, Javier Pozueta-Romero

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**TITLE:** *Escherichia coli* glycogen metabolism is controlled by the PhoP-PhoQ regulatory system at submillimolar environmental  $Mg^{2+}$  concentrations, and is highly interconnected with a wide variety of cellular processes

**Authors:** Manuel Montero\*, Gustavo Eydallin\*, Alejandro M. Viale, Goizeder Almagro, Francisco J. Muñoz, Mehdi Rahimpour, María T. Sesma, Edurne Baroja-Fernández and Javier Pozueta-Romero

**Affiliation:** Instituto de Agrobiotecnología (CSIC, UPNA, Gobierno de Navarra). Mutiloako etorbidea zenbaki gabe, 31192 Mutiloabeti, Nafarroa, Spain.

**Key words:** AMP, energy status, Keio collection,  $Mg^{2+}$ , stringent response

**Abbreviations:** ADPG, ADP-glucose; cAMP, cyclic AMP; COG, cluster of orthologous groups; CRP, cAMP receptor protein; GlgA, glycogen synthase; GlgB, branching enzyme; GlgP, glycogen phosphorylase; GlgX, debranching enzyme; G6P, glucose-6-phosphate; ppGpp, guanosine 5'-diphosphate 3'-diphosphate; WT, wild type

**Corresponding author:** Javier Pozueta-Romero.

Address: Instituto de Agrobiotecnología. Mutiloako etorbidea zenbaki gabe, 31192 Mutiloabeti, Nafarroa, Spain. E-mail: [javier.pozueta@unavarra.es](mailto:javier.pozueta@unavarra.es). Phone: (34) 948168009. FAX: (34) 948232191

**Footnote:** A. M. V. present address is "Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET), Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina". \* M. M. and G. E. have equally contributed to this work.

**Short title:** Control of *E.coli* glycogen metabolism by  $Mg^{2+}$

## SYNOPSIS

Using the Keio collection of gene-disrupted mutants of *Escherichia coli* we have recently carried out a genome-wide screening of the genes affecting glycogen metabolism. Among the mutants identified in the study,  $\Delta$ mgfA,  $\Delta$ phoP and  $\Delta$ phoQ cells, all lacking genes that are induced under low extracellular  $Mg^{2+}$  conditions, displayed glycogen-deficient phenotypes. In this work we show that these mutants accumulated normal glycogen levels when the culture medium was supplemented with submillimolar  $Mg^{2+}$  concentrations. Expression analyses conducted in wild type,  $\Delta$ phoP and  $\Delta$ phoQ cells showed that the *glgCAP* operon is under PhoP-PhoQ control in the submillimolar  $Mg^{2+}$  concentration range. Subsequent screening of the Keio collection under non-limiting  $Mg^{2+}$  allowed the identification of 183 knock-out mutants with altered glycogen levels. The stringent and general stress responses, end-turnover of tRNA, intracellular AMP levels, and metabolism of amino acids, iron, carbon and sulfur were major determinants of glycogen levels. *glgC::lacZY* expression analyses using mutants representing different functional categories revealed that the *glgCAP* operon belongs to the RelA regulon. We propose an integrated metabolic model wherein glycogen metabolism is (a) tightly controlled by the energy and nutritional status of the cell and (b) finely regulated by changes in environmental  $Mg^{2+}$  occurring at the submillimolar concentration range.

## INTRODUCTION

Glycogen is a branched homopolysaccharide of  $\alpha$ -1,4-linked glucose subunits with about 5%  $\alpha$ -1,6-linked glucose at the branching points. The exact role of this polyglucan in prokaryotes is still unknown, although several works have linked glycogen metabolism to environmental survival and even to colonization and virulence in the case of pathogens [1-5]. Synthesized by glycogen synthase (GlgA) using ADP-glucose (ADPG) as the glucosyl moiety donor, glycogen accumulation in *Escherichia coli* and *Salmonella spp.* occurs when cellular carbon sources are in excess under conditions of other nutrients deficiency [6,7]. Under these conditions synthesis of proteins and nucleic acids is arrested, and ATP and excess carbon are diverted towards glycogen biosynthesis.

Regulation of glycogen biosynthesis in *E. coli* involves a complex and still not well defined assemblage of factors. At the level of enzyme activity for instance, the glycogen biosynthetic process is subjected to the allosteric regulation of GlgC, a protein that catalyzes the production of ADPG from ATP and glucose-1-phosphate [8,9]. At the level of gene expression, *in vitro* and *in vivo* experimental evidences suggested that the process depends on the regulation of two contiguous operons, *glgBX* (encompassing the genes coding for glycogen branching (GlgB) and debranching (GlgX) enzymes), and *glgCAP* (encoding the GlgC and GlgA anabolic enzymes as well as the catabolic glycogen phosphorylase (GlgP)) [8,10,11]. An additional gene, *glgS*, regulates glycogen synthesis in a still undefined manner [12].

Transcriptome analyses of *E. coli* cells growing on either glucose-containing minimal or rich media have shown that all *glg* genes are upregulated when cultures enter the late logarithmic growth phase [13]. Experimental evidences provided by different authors have indicated that the *glgCAP* operon is positively regulated at the transcriptional level by the guanosine 5'-diphosphate 3'-diphosphate (ppGpp) stringent response regulator synthesized by the product of *relA* [10,14-17], and by the cyclic AMP (cAMP)/cAMP receptor protein (CRP) complex ([18-20], however, for an opposite view see [12]). At the post-transcriptional level, glycogen accumulation in *E. coli* is negatively affected by the central carbon storage regulator CsrA, which prevents *glgC* translation [21-23]. In addition, glycogen accumulation is positively influenced by the general stress factor RpoS by a still undefined mechanism [12].

To obtain clues on the role(s) of glycogen metabolism and its connection with other biological process, we have recently carried out a genome-wide screening of the genes affecting glycogen metabolism [7] using a systematic and comprehensive gene-disrupted *E. coli* mutant collection (the Keio collection [24]). This study identified 65 genes whose mutations substantially affected cell glycogen content. Amongst different mutants with reduced glycogen content we identified those lacking *phoP*, *phoQ* and *mgtA*. This observation was particularly interesting since these genes are induced under low extracellular  $Mg^{2+}$  concentration conditions [25,26]. In fact, PhoP-PhoQ is a two-component regulatory system occurring in *E. coli* and *Salmonella spp.* that monitors the availability of extracellular  $Mg^{2+}$ , and transcriptionally controls the expression of many genes, including *mgtA* which codes for an energy-dependent  $Mg^{2+}$  import system [27,28]. The overall data thus suggested that extracellular  $Mg^{2+}$  could represent an important determinant of glycogen accumulation in *E. coli*.

$Mg^{2+}$  is the most abundant divalent cation within living cells, being a stabilizing factor for membranes, tRNA, ribosomes, etc. that strongly determines cell metabolic and energetic status [25]. In fact, the balance between  $Mg^{2+}$ -bound and  $Mg^{2+}$ -free adenylates is a major determinant of the correct functioning of many enzymes. This balance depends on factors such as total content of intracellular adenylates and free

Mg<sup>2+</sup>, adenylate kinase (Adk) activity, membrane potential and transport of extracellular Mg<sup>2+</sup> across membranes [25,29,30]. To investigate the influence of extracellular Mg<sup>2+</sup> on *E. coli* glycogen metabolism in this work we have measured both ATP and glycogen contents, and expression of *glgC* in cells cultured in glucose Kornberg medium without and with Mg<sup>2+</sup> supplementation. We found that changes in external Mg<sup>2+</sup> concentrations in the submillimolar range have profound effects on the ability of *E. coli* to accumulate glycogen. We have therefore re-screened the Keio collection for glycogen altered phenotypes employing culture medium supplemented with Mg<sup>2+</sup>. With the overall data amassed in this work we conclude that synthesis and utilization of glycogen is under control of a complex and intricate network wherein cell energy and nutritional status play crucial roles. We propose an integrated metabolic model wherein glycogen (a) is highly interconnected with a number of cellular processes and (b) is finely regulated by changes in environmental Mg<sup>2+</sup> occurring at the submillimolar concentration range. In addition, based in our observations we propose that intracellular AMP levels are critically important in regulating glycogen biosynthesis.

## EXPERIMENTAL PROCEDURES

### *E. coli K-12 mutants and culture conditions*

We used mutants from the systematic, single-gene knockout mutant collection of the nonessential genes of the *E. coli* K-12 derivative BW25113 [24]. For quantitative measurement of glycogen content, cells were grown at 37°C with rapid gyratory shaking in liquid Kornberg medium (1.1% K<sub>2</sub>HPO<sub>4</sub>, 0.85% KH<sub>2</sub>PO<sub>4</sub>, 0.6% yeast extract from Duchefa, Haarlem, The Netherlands) containing 250 µM Mg<sup>2+</sup> and 50 mM glucose after inoculation with 1 volume of an overnight culture for 50 volumes of fresh medium. Cells from cultures entering the stationary phase were centrifuged at 4,400 x g for 15 min, rinsed with fresh Kornberg medium, resuspended in 40 mM Tris-HCl (pH 7.5) and disrupted by sonication prior to quantitative glycogen measurement (see below). Solid Kornberg medium was prepared by addition of 1.8% bacteriological agar to liquid Kornberg medium before autoclaving.

### *Screening for mutants with altered glycogen content*

First screening of glycogen mutants on solid glucose Kornberg medium containing 250 µM Mg<sup>2+</sup> was carried out following the glycogen iodine staining method [31]. Mutants identified using this procedure were subsequently cultured in liquid glucose Kornberg medium containing 250 µM Mg<sup>2+</sup>, and subjected to quantitative glycogen measurement analyses using an amyloglucosidase/hexokinase/glucose-6P dehydrogenase-based test kit from Sigma. Intracellular glycogen content was referred to protein, which was measured using a Bio-Rad prepared reagent. Mutants with altered glycogen content were further confirmed for the indicated deletions by PCR using specific primers. The gene mutations promoting altered glycogen accumulation described in this work imply that both insertional mutants for each individual gene provided in the Keio collection displayed the indicated phenotype. The function of each gene whose deletion affects glycogen accumulation was assigned by referring to the EchoBASE (<http://ecoli-york.org/>) and EcoCyc (<http://www.ecocyc.org/>) databases.

### *Adenine nucleotide extraction and measurement*

AMP, ADP and ATP were extracted and measured essentially as described in [31]. Cells (ca. 2.5 g fresh weight) were collected at the onset of the stationary phase, treated with 1 ml of 1 M HClO<sub>4</sub>, left on ice for 1 h and centrifuged at 10,000 x g for 15 min. The supernatant thus obtained was neutralized with K<sub>2</sub>CO<sub>3</sub>, centrifuged at 10,000 x g and adenylates were measured by HPLC on a system obtained from P.E. Waters and Associates fitted with a Partisil-10-SAX column.

### *Determination of yeast extract metal contents*

The analysis of the mineral contents of the commercial yeast extract used in this work (Duchefa, Haarlem, The Netherlands, lot 2782/01) was done by Inductively Coupled Plasma (ICP) Spectrometry using a Varian Vista-MPX ICP. The corresponding contents (in mg/kg dry weight) were: Mg, 182.72 ±7.06; Mn, 0.53 ±0.02; Co, 1.45 ±0.02; Ca, 300.39 ±5.04; Fe, 32.73 ±1.82; and S, 6931 ±71.

### *Production of *phoQ*-, *purB*- and *glgA*- expressing cells*

Cells expressing *phoQ*, *purB* and *glgA* in trans were obtained by incorporation of *phoQ*, *purB*- and *glgA*-expression vectors from the ASKA library [32] into electrocompetent cells.

### ***LacZY transcriptional fusions***

The kanamycin resistance cassette inserted at the deletion point of *E. coli* genes from Keio collection was removed by using temperature-sensitive plasmid pCP20 carrying the FLP recombinase [33]. The scar sequence left after removal of the resistance cassette included a 34-nucleotide FRT site [24], which was used to build *lacZY* transcriptional fusions as reported in [34]. Briefly, Keio collection *E. coli* mutants carrying pCP20 plasmid were transformed with pKG137, which has functional *lacZY* and Km<sup>R</sup> cassette that integrated in the proper orientation at the FRT site by the action of the FLP recombinase, yielding to *lacZY* transcriptional fusions where the original resistance cassette of the Keio collection was previously placed.

### ***Western blot analyses***

Bacterial extracts were separated by 10% SDS-PAGE, transferred to nitrocellulose filters, and immunodecorated by using the antisera raised against GlgC [35] and a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma).



## RESULTS AND DISCUSSION

### ***Extracellular $Mg^{2+}$ is an important determinant of *E. coli* glycogen accumulation***

Among different mutants of the Keio collection with altered glycogen content identified in our previous work [7],  $\Delta mgtA$ ,  $\Delta phoP$  and  $\Delta phoQ$  cells displayed glycogen-deficient phenotypes. Because the expression of *mgtA*, *phoP* and *phoQ* is a PhoP-PhoQ-dependent process triggered under conditions of low extracellular  $Mg^{2+}$ , our previous observations suggested that (a) the glucose Kornberg culture medium employed in our previous work contained limiting  $Mg^{2+}$  concentrations, and (b) environmental  $Mg^{2+}$  may represent an important determinant of glycogen metabolism in *E. coli*. To test these possibilities we measured the  $Mg^{2+}$  content in the glucose Kornberg culture medium employed in our previous work. Furthermore, because *mgtA* is a reporter gene of low external and cytoplasmic  $Mg^{2+}$  concentrations [28], we also analyzed the expression of chromosomal *mgtA::lacZY* transcriptional fusion constructed on BW25113 wild type (WT) cells when cultured in glucose Kornberg medium without and with  $Mg^{2+}$  supplementation. Finally, we measured the glycogen content in WT,  $\Delta mgtA$ ,  $\Delta phoP$ , and  $\Delta phoQ$  cells cultured under the above conditions.

Determination of different metal contents in the yeast extract used for the preparation of the glucose Kornberg medium used in this and in our previous work [7] indicated the presence of around 50  $\mu M$   $Mg^{2+}$ . Although this intrinsic concentration would be expected to suffice for an active bacterial growth [25], it was actually limiting for growth of the BW25113 strain used here, as judged by comparing both the growth rates and yields obtained with and without supplementation of submillimolar  $Mg^{2+}$  concentrations (**Supplemental Figure 1**). Limitations in bacterial growth due to deficient  $Mg^{2+}$  contents are most probably due to the complexing effect exerted by the high phosphate concentration (ca. 120 mM) of the Kornberg medium [36]. Analyses of the expression of a chromosomal *mgtA::lacZY* transcriptional fusion on WT cells at the onset of the stationary phase revealed that *mgtA* was highly expressed at the onset of the stationary phase in medium lacking  $Mg^{2+}$  supplementation (starting  $Mg^{2+}$  concentration of 50  $\mu M$ ), while its expression was strongly repressed when the starting  $Mg^{2+}$  concentration reached 250  $\mu M$  or higher (**Supplemental Figure 2**). The overall data thus showed that the culture medium employed in our previous work [7] reached a threshold limiting  $Mg^{2+}$  concentration during bacterial growth.

Supplementation of the culture medium with submillimolar concentrations of  $Mg^{2+}$  substantially increased glycogen content in cells at the onset of the stationary phase, almost doubling the amount of this polyglucan when the starting concentration of  $Mg^{2+}$  reached 250  $\mu M$  or higher (not shown). This enhancing effect of  $Mg^{2+}$  on glycogen accumulation was even more marked in  $\Delta phoP$ ,  $\Delta phoQ$ , and  $\Delta mgtA$  cells (and in particular for the first two mutants, **Figure 1**), the final amount of glycogen accumulated at the onset of the stationary phase being similar for all cell types when cultured under non-limiting  $Mg^{2+}$  concentrations. The overall data thus showed that environmental  $Mg^{2+}$  concentration changes in the submillimolar range may represent important determinants of glycogen accumulation in *E. coli*.

### ***Extracellular $Mg^{2+}$ highly determines the intracellular levels of ATP in *E. coli****

$Mg^{2+}$  is the most abundant divalent cation within cells, its intracellular concentration strongly determining the cell metabolic and energy status [25]. Since ATP acts as a substrate for reactions directly and indirectly involved in glycogen biosynthesis, we found of interest to analyze the intracellular ATP content in WT bacteria entering the stationary phase either in limiting and non-limiting  $Mg^{2+}$  concentrations (50  $\mu M$   $Mg^{2+}$



and 250  $\mu\text{M}$   $\text{Mg}^{2+}$ , respectively). As shown in **Figure 2**, these analyses revealed that ATP content in cells cultured under non-limiting  $\text{Mg}^{2+}$  conditions was, in average, 2.5-fold higher than that observed for cells cultured under limiting  $\text{Mg}^{2+}$  conditions. The overall data thus indicate that the reduced glycogen contents observed in *E. coli* cells subjected to limiting  $\text{Mg}^{2+}$  concentrations (cf. **Figure 1**) could be ascribed, at least in part, to reduced intracellular levels of ATP.

***Expression of E. coli glgCAP operon is positively controlled by the PhoP-PhoQ regulatory system under limiting  $\text{Mg}^{2+}$  conditions***

We next investigated whether expression of the *E. coli glgCAP* operon is subjected to extracellular  $\text{Mg}^{2+}$  regulation. For this purpose we constructed chromosomal *glgC::lacZY* gene transcriptional fusions in WT,  $\Delta\text{mgtA}$ ,  $\Delta\text{phoQ}$  and  $\Delta\text{phoP}$  cells, and compared the  $\beta$ -galactosidase activity levels reached at different growth times of these bacteria when cultured under both limiting and non-limiting  $\text{Mg}^{2+}$  conditions (50  $\mu\text{M}$  and 1 mM  $\text{Mg}^{2+}$ , respectively). We also determined by western blot analyses the contents of GlgC at the onset of the stationary phase in WT,  $\Delta\text{mgtA}$ ,  $\Delta\text{phoQ}$  and  $\Delta\text{phoP}$  cells when growing under the two different  $\text{Mg}^{2+}$  concentration regimes.

Growth of  $\Delta\text{mgtA}$ ,  $\Delta\text{phoQ}$  and  $\Delta\text{phoP}$  cells, all impaired in the MgtA high affinity  $\text{Mg}^{2+}$  import system induced under low environmental  $\text{Mg}^{2+}$  concentrations [25,28], was reduced under limiting  $\text{Mg}^{2+}$  concentration conditions when compared with WT cells (**Figure 3A**). Supplementation of the culture medium with 1 mM  $\text{Mg}^{2+}$  restored growth of the above mutants to WT levels (**Figure 3C**). Analysis of  $\beta$ -galactosidase activity levels derived from *glgC::lacZY* transcriptional fusions indicated that, under limiting  $\text{Mg}^{2+}$  concentration conditions, *glgC* expression was significantly lower in both  $\Delta\text{phoP}$  cells and  $\Delta\text{phoQ}$  cells than in both WT and  $\Delta\text{mgtA}$  cells (**Figure 3B**). Moreover, supplementation of the culture medium with 1 mM  $\text{Mg}^{2+}$  largely restored *glgC::lacZY* expression in  $\Delta\text{phoP}$  and  $\Delta\text{phoQ}$  cells (**Figure 3D**), the overall data indicating that expression of the *E. coli glgCAP* operon is under control of the PhoP-PhoQ system under low environmental  $\text{Mg}^{2+}$  conditions. In agreement with this proposal, western blot analysis of GlgC showed that both  $\Delta\text{phoP}$  and  $\Delta\text{phoQ}$  cells accumulated lower levels of this protein than WT cells when cultured under limiting  $\text{Mg}^{2+}$  concentrations (**Figure 3E**), whereas GlgC levels in  $\Delta\text{phoP}$  and  $\Delta\text{phoQ}$  cells were comparable to those of WT cells when cultured in non-limiting  $\text{Mg}^{2+}$  conditions (**Figure 3E**). Furthermore, expression of *phoQ* from a plasmid vector in  $\Delta\text{phoQ}$  cells cultured under limiting  $\text{Mg}^{2+}$  concentrations resulted in WT levels of GlgC (**Figure 3E**). GlgC levels in  $\Delta\text{mgtA}$  cells impaired in the MgtA high affinity  $\text{Mg}^{2+}$  import system were similar to those of WT cells when cultured in both limiting and non-limiting  $\text{Mg}^{2+}$  concentration conditions (not shown). These observations, together with those showing WT expression levels of *glgC::lacZY* in  $\Delta\text{mgtA}$  cells (cf. **Figure 3B**), rule out a significant role of low intracellular  $\text{Mg}^{2+}$  concentrations in the induction of *glgCAP* expression, and reinforce the notion that this operon is under positive control of the PhoP-PhoQ regulatory system under limiting environmental  $\text{Mg}^{2+}$  concentrations. Noteworthy, sequence analyses of the 1000-bp-long promoter region upstream from the ATG initiation codon of *glgC* did not reveal the presence of a putative PhoP box, defined in *E. coli* as (T)G(T)TT(AA) [37] or (T/G)GTTTA [26] tandem direct repeats. It is thus possible that the PhoP-PhoQ mediated  $\text{Mg}^{2+}$  regulation of *glgCAP* is indirect, as has been described for the PmrA-regulated genes [38].

Because  $\text{Mg}^{2+}$  is a determinant of both intracellular ATP levels and correct functioning of many enzymes including those involved in glycogen biosynthesis, the

moderate reduction of glycogen levels occurring in *ΔmgtA* cells cultured under limiting (50 μM)  $Mg^{2+}$  concentration (60% of the glycogen accumulated by WT cells, cf. **Figure 1** and Figure 2 in [7]) can be ascribed to both low ATP production and reduced activity of enzymes directly or indirectly involved in glycogen metabolism. In turn, the more drastically reduced glycogen levels of *ΔphoP* cells cultured with 50 μM  $Mg^{2+}$  (10% of the levels occurring in WT cells, cf. **Figure 1** in this work and Figure 2 in [7]) can be ascribed to a combination of factors including reduced ATP levels, low activity of enzymes involved in glycogen metabolism, and low expression of *glgCAP* operon.

### ***Screening, identification and classification of E. coli genes whose deletions affect glycogen accumulation under non-limiting $Mg^{2+}$ conditions***

Using the iodine staining technique we screened the Keio mutant collection for altered glycogen content when cells were cultured in glucose Kornberg medium containing a starting  $Mg^{2+}$  concentration of 250 μM. In the presence of iodine vapors, “glycogen-excess” mutants stain darker than their brownish parental cells, whereas “glycogen-deficient” mutants stain yellow (**Supplemental Figure 3**). On inspecting the mutant library, 92 mutants (2.3 % of the library) showed increased levels of glycogen (**Figure 4**), while 91 mutants (2.3 % of the library) had reduced levels of glycogen when compared to WT cells (**Figure 5**). The 183 genes now identified whose deletions affect glycogen accumulation were classified into clusters of orthologous groups (COGs) categories (**Tables 1** and **2**). **Supplemental Table 1** shows the function of each gene product. Notably, a large group of 31 mutants, representing 17 % of the genes identified, corresponded to genes of unknown function, about which little or nothing is known.

As summarized in **Figure 6**, the general trend indicates that glycogen metabolism of *E. coli* cells cultured under non-limiting  $Mg^{2+}$  conditions is affected by proteins that can be embodied in the following groups:

- Stringent response,
- General stress response,
- Amino acid metabolism,
- Proteases,
- Carbon source sensing, transport and metabolism,
- Iron metabolism, (\*)
- Energy production,
- Cellular redox status, (\*)
- Envelope composition and integrity,
- Osmotic stress, (\*)
- Nucleotide metabolism, (\*)
- End-tRNA turnover (\*)

The asterisk (\*) indicates categories which were not identified in our previous screening [7].

In the following sections, the possible roles of some of the identified components are discussed.

### ***The stringent response***

During nutrient starvation *E. coli* elicits the so-called stringent response that switches the cell from a growth-related mode to a maintenance/survival/biosynthesis mode. The hallmark of this pleiotropic response is the accumulation of the alarmone (p)ppGpp, which is synthesized by the *relA* product [39]. When (p)ppGpp accumulates, growth is

arrested and it is expected that an important pool of ATP will be diverted towards glycogen biosynthesis under conditions of carbon source excess. Consistent with this view, *ΔrelA* cells of the Keio collection impaired in (p)ppGpp synthesis displayed a glycogen-deficient phenotype (**Figure 5**).

Although it is widely accepted that glycogen accumulation is positively influenced by *relA* [14,21], available data on the regulatory action of this gene on the expression of *glg* genes are contradictory. Whereas transcription profiling analyses of the stringent response failed to show that *glg* genes belong to the RelA regulon [41], similar type of analyses showed that *relA* positively regulates their expression [17]. Our own analyses of the expression of a chromosomal *glgC::lacZY* gene fusion in both WT cells and *ΔrelA* cells of the Keio collection indicated that *glgCAP* expression was largely abolished in cells lacking *relA* (**Figure 7**), strongly indicating that the *E. coli glgCAP* operon belongs to the RelA regulon.

*E. coli* glycogen accumulation could be subjected also to different levels of post-transcriptional regulation by (p)ppGpp. In addition to its role as a global regulator of gene expression in bacteria, (p)ppGpp potently inhibits PurA [42], which catalyzes the first committed step in *de novo* biosynthesis of the main GlgC inhibitor, AMP [9]. Noteworthy, *ΔrelA* cells accumulated high levels of AMP when compared to WT cells (**Figure 8**). It is thus conceivable that reduction of glycogen levels in *ΔrelA* cells is due, at least in part, to the inhibitory effect exerted by high cellular levels of AMP on GlgC. This view is also consistent with our observations that *ΔpurA* cells accumulated low levels of AMP (**Figure 8**) and high levels of glycogen (**Figure 4**) when compared to WT cells, and with previous reports showing that cells bearing a mutated GlgC form that is insensitive to AMP allosteric regulation displayed glycogen-excess phenotypes [36].

The overall data thus indicate that *relA* control on *E. coli* glycogen levels may result from an interplay of its (p)ppGpp-mediated positive effect on *glgCAP* transcription, deviation of surplus ATP flux from nucleic acid and protein biosynthetic pathways to glycogen production, and its negative effect on intracellular levels of the GlgC negative effector AMP.

### ***The general stress response***

RpoS is an alternative sigma factor of the RNA polymerase for the general stress response, which has been proposed to be absolutely required for normal glycogen biosynthesis [43]. *glgA::lacZ* and *glgC::lacZ* fusions analyses have shown that RpoS does not regulate *glgCAP* transcription in the MC4100 strain [12], but positively controls the expression of *glgS*, a gene whose product exerts a positive but still unknown effect on glycogen accumulation [12]. Our analysis using *glgC::lacZY* fusions constructed on *ΔrpoS* mutants of the Keio collection also indicated no significant effect of this mutation on the expression of the *glgCAP* operon in cells grown in glucose Kornberg medium (**Figure 7**).

RpoS is regulated in *E. coli* in response to several different signals. At the transcriptional level, (p)ppGpp positively affects *rpoS* transcript elongation and/or stability [44]. At the post-transcriptional level full *rpoS* expression requires *hfq* function [45], DksA [40], and DnaK [46]. Consistent with the view that RpoS exerts a positive effect on glycogen accumulation, *ΔrpoS* cells of the Keio collection accumulated low glycogen levels when compared to WT cells (**Figure 5**). In addition, *ΔdksA*, *Δhfq*, *ΔrelA*, *ΔdnaK* and *ΔglgS* cells displayed glycogen-deficient phenotypes (**Figure 5**).

### ***Amino acid metabolism***

Yeast extract (the amino acid source of the Kornberg medium employed in this work) is deficient in various amino acids [47]. Amino acid starvation elicits the stringent response mediated by (p)ppGpp [40]. It is thus highly conceivable that mutations impairing amino acid synthesis and/or provision will lead to glycogen over-accumulation. Consistent with this view, several mutants impaired in the synthesis of tyrosine, phenylalanine, tryptophan and arginine ( $\Delta aroA$ ,  $\Delta aroC$ ,  $\Delta aroF$ ,  $\Delta aroK$ ,  $\Delta aroL$ ,  $\Delta argH$ ,  $\Delta carA$ ,  $\Delta carB$ , and  $\Delta tyrA$ ) or in proline import ( $\Delta proW$ ) accumulated higher levels of glycogen than the WT strain (**Figure 4**). In addition, several mutants impaired in either the import of sulfur sources ( $\Delta cysA$ ,  $\Delta cysP$ ,  $\Delta cysU$  and  $\Delta cysW$ ) or enzymatic activities required for cysteine biosynthesis ( $\Delta cysC$ ,  $\Delta cysD$ ,  $\Delta cysE$ ,  $\Delta cysG$ ,  $\Delta cysH$ ,  $\Delta cysI$ ,  $\Delta cysJ$ ,  $\Delta cysM$ ,  $\Delta cysN$ ,  $\Delta serA$ ,  $\Delta serB$  and  $\Delta serC$ ) also displayed glycogen-excess phenotypes (**Figure 4**). Because cysteine constitutes the almost exclusive metabolic entrance of reduced sulfur into cell metabolism, it is likely that the glycogen-excess phenotype of mutants impaired in cysteine biosynthesis is the result of the stringent response elicited by both nitrogen (amino acid) and sulfur starvation.

It is important to note that, consistent with the view that the stringent response downregulates the production of AMP (see above), glycogen-excess mutants impaired in amino acid synthesis such as  $\Delta serA$  and  $\Delta cysI$  accumulated low AMP levels (**Figure 8**). It is thus conceivable that, as discussed above, increase of glycogen levels in these mutants may also be ascribed to high GlgC activity occurring as a consequence of the low intracellular concentrations of the GlgC negative effector AMP.

### ***Proteases***

Lon and the two-component Clp ATP-dependent proteases play a major role in the degradation of damaged polypeptides and in the recycling of amino acids in response to nutritional downshift, which is a process involving a major portion of the maintenance energy requirement [48]. Therefore, it is expected that impairments in major cell protein degradation machineries will elicit the stringent response that, as discussed above, lead to enhancement of glycogen content. Consistent with this presumption,  $\Delta lon$ ,  $\Delta clpP$  or  $\Delta clpA$  cells accumulated higher levels of glycogen than WT cells (**Figure 4**).

### ***Carbon sensing, transport and metabolism***

As expected,  $\Delta pgm$ ,  $\Delta glgC$  and  $\Delta glgA$  cells (all lacking enzymes directly implicated in glycogen synthesis) displayed glycogen-less phenotypes (**Figure 5**). Because *E. coli* possesses sources, other than GlgC, of ADPG linked to glycogen biosynthesis [31,35], the total absence of glycogen in  $\Delta glgC$  cells is ascribed to the polar effect of *glgC* deletion on the downstream *glgA* gene. This presumption was confirmed using  $\Delta glgC$  cells ectopically expressing *glgA* since, as shown in **Supplemental Figure 4**, these cells accumulated high glycogen levels.  $\Delta glgB$  cells accumulated very low levels of glycogen (**Figure 5**) and, due to their disability to produce branched  $\alpha$ -1,6-glucosidic linkages, they stained blue when exposed to iodine vapors (**Supplemental Figure 3**) [49]. The very low glycogen content occurring in  $\Delta glgB$  cells is ascribed to the fact that (a) GlgB is likely required for the initiation of the glycogen granule [50] and/or (b) formation of  $\alpha$ -1,6-glucosidic may be absolutely required for maximal glycogen synthesis.  $\Delta glgX$  and  $\Delta glgP$  cells showed a marked increase of glycogen levels, which is in agreement with the role of GlgX and GlgP in glycogen breakdown [11,51].  $\Delta ptsI$ ,  $\Delta ptsH$  and  $\Delta ptsG$  cells displayed glycogen-deficient phenotypes (**Figure 5**), which is consistent



with the overriding importance of the phosphoenolpyruvate dependent phosphotransferase system (PTS) in the sensing and uptake of extracellular glucose, and in reflecting the energy status of the cell [52].

Both cAMP produced by the membrane-bound adenylate cyclase (the product of *cya*), and CRP are required for expression of *glgS* and PTS-related genes required for normal glycogen production [12,53]. Consistent with previous reports showing that *cya* and *crp* are important regulators of glycogen metabolism [54],  $\Delta cya$  and  $\Delta crp$  cells of the Keio collection displayed marked glycogen-deficient phenotypes (**Figure 5**). Therefore, the glycogen-deficient phenotype of  $\Delta cya$  and  $\Delta crp$  cells could be ascribed, at least in part, to downregulation of *glgS* and/or PTS-related genes. As to the possible regulation of *glg* genes by *cya* and *crp*, both *in vitro* and *in vivo* experiments have shown that cAMP/CRP positively regulates *glgC* and *glgA* expression in *E. coli* [10,16,20]. However, recent transcriptome analyses failed to indicate that *glg* genes belong to the cAMP regulon [53]. Our own analysis using a chromosomal *glgC::lacZY* fusion constructed on the  $\Delta cya$  mutant of the Keio collection (**Figure 7**) revealed that expression of *glgCAP* operon was not affected in this mutant, reinforcing the idea that *E. coli* glycogen metabolism is not regulated at the level of *glgCAP* expression by cAMP. We must emphasize that, by still unidentified mechanisms and reasons,  $\Delta cya$  and  $\Delta crp$  cells accumulated high levels of AMP when compared to WT cells (**Figure 8**). The overall data thus indicate that control of *E. coli* glycogen metabolism by cAMP is the result of an interplay between the positive effect of this cyclic nucleotide on expression of *glgS* and PTS-related genes, and its negative effect on the intracellular levels of the GlgC negative effector AMP.

$\Delta pgi$  cells lack phosphoglucose isomerase, the enzyme that catalyzes the conversion of glucose-6-phosphate (G6P) into fructose-6-phosphate. As shown in **Figure 5**  $\Delta pgi$  cells accumulated low glycogen levels. In glucose-containing media  $\Delta pgi$  cells accumulate high levels of G6P [55]. This hexose-P strongly inhibits the glucose transporter and destabilizes *ptsG* mRNA [55,56]. Therefore, the glycogen-deficient phenotype of  $\Delta pgi$  cells may be ascribed to impairments in the incorporation of glucose linked to glycogen biosynthesis as a consequence of the high intracellular G6P levels.

$\Delta ybhE$  and  $\Delta zwf$  cells lack the two enzymes of the pentose phosphate pathway 6-phosphogluconolactonase and G6P dehydrogenase, respectively. Metabolic intermediates of this pathway are necessary for the production of aromatic amino acids. It is therefore conceivable that, as discussed above, the glycogen-excess phenotype displayed by these mutants (**Figure 4**) is the consequence of the stringent response triggered by the lack of internal amino-acid supply.

### Iron metabolism

Iron is needed as a cofactor for a large number of enzymes. In aerobic conditions *E. coli* utilizes high-affinity extracellular siderophores that solubilize and capture Fe(III) prior to transport and metabolism [57]. Fur, a dominant sensor of iron availability, generally represses iron siderophore biosynthetic and transport genes such as *fepB*, *fepD* and *fepG* [57]. Under iron limiting conditions, iron dissociates from Fur, and increased transcription of genes ensues. Iron limitation causes the stringent response due to a SpoT-dependent increase of the ppGpp pool [58]. It is therefore expected that impairment in iron supply will lead to a stringent response induced glycogen over-accumulation (see above). Consistent with this presumption,  $\Delta fepB$ ,  $\Delta fepD$  and  $\Delta fepG$  cells defective in the transport of ferric-siderophore displayed glycogen-excess phenotypes (**Figure 4**). By contrast,  $\Delta fur$  cells accumulated low levels of glycogen when compared with WT cells (**Figure 5**). In addition to its role as sensor of iron

availability, Fur can be considered to be a global regulator [57]. To investigate the possible Fur-dependent transcriptional regulation of glycogen metabolism we analyzed *glgCAP* expression in  $\Delta fur$  cells of the Keio collection by means of a chromosomal *glgC::lacZY* fusion. As shown in **Figure 7**, *glgC::lacZY* derived  $\beta$ -galactosidase activity was not substantially modified in the  $\Delta fur$  mutants as compared to WT cells, indicating that *E. coli glg* genes are not members of the Fur regulon.

#### **Energy production and cellular redox status**

ATP is a primary signal in regulating glycogen biosynthesis, and acts as substrate for the ADPG-producing reaction catalyzed by GlgC [56]. It is therefore conceivable that mutations in components required for the proper functioning of the aerobic electron transport chain and ATP generation will negatively affect glycogen accumulation. Consistent with this view,  $\Delta ubiG$  and  $\Delta ubiH$  cells (deficient in ubiquinone production) and  $\Delta iscU$ ,  $\Delta iscS$ ,  $\Delta fdx$  and  $\Delta hscB$  cells impaired in the machinery for the assembly/maintenance of Fe-S clusters (components required for the proper functioning of the aerobic electron transport chain and ATP generation [59]) showed reduced glycogen levels (**Figure 5**).

Glutathione is a major determinant of the redox status of the cell, playing a prime role in maintaining the correct assembly of electron transport chain components [60].  $\Delta gshB$  and  $\Delta gor$  cells (lacking the machinery necessary to produce and reduce glutathione, respectively) displayed a glycogen-deficient phenotype (**Figure 5**), indicating that some components involved in glycogen metabolism are strongly affected by the cellular redox status.

#### **Envelope composition and integrity**

RpoE is an essential transcription initiation factor that governs the response to envelope stress and the expression of genes that are needed to heal envelope damage. The major point of regulation of RpoE is at the level of its interaction with the anti-sigma RseA factor [61]. When *E. coli* is subjected to extra-cytoplasmic stresses, RseA degrades and RpoE activity is induced. Another major point of regulation of RpoE takes place at the post-transcriptional level, since it has been shown that Hfq interaction with *rseA* mRNA downregulates *rseA* expression [62]. Remarkably both  $\Delta rseA$  and  $\Delta hfq$  cells accumulated low glycogen levels (**Figure 5**), suggesting that RpoE-mediated envelope stress response may to some extent negatively affect glycogen accumulation.

Mutants of genes coding for proteins involved in the maintenance of the cell envelope integrity such as *rfaE*, *galU*, *tolB*, *tolR*, *tolQ*, *pal*, and *ponB*, displayed glycogen-deficient phenotypes (**Figure 5**). All these mutants are likely to promote both envelope stress and deformation of membrane that causes inhibition of the electron transport chain, energy production and formation of membrane potential necessary for nutrient import [63,64]. The  $\Delta rfaE$  mutant for instance lacks an enzyme required for *E. coli* lipopolysaccharide biosynthesis [65].  $\Delta galU$  lacks the enzyme that catalyses the synthesis of UDP-glucose necessary for the synthesis of cell envelope components [66]. Moreover,  $\Delta tolB$ ,  $\Delta tolR$ ,  $\Delta tolQ$  and  $\Delta pal$  cells are impaired in proteins of the Tol-Pal system essential in maintaining envelope integrity [67]. In addition,  $\Delta ponB$  ( $\Delta mrcB$ ) cells lack a bi-functional membrane-bound enzyme catalyzing transglycosylation and transpeptidation reactions, which are essential in the late stages of peptidoglycan biosynthesis [68].



### Osmotic stress

Osmotic upshock results in a large decrease in the cytoplasmic volume and deformation of membrane that causes inhibition of electron transport system, respiration, sugar uptake and glycogen accumulation [63,69,70]. In addition, osmotic stress induces the expression of RpoE [71]. Consistent with the view that the energy status of the cell and glycogen metabolism are strongly determined by the cell osmotic conditions, *ΔkdpB* cells impaired in the high affinity potassium influx system that maintains the turgor pressure across the inner membrane [72] displayed a glycogen-deficient phenotype (**Figure 5**).

### Nucleotide metabolism

AMP is the natural negative regulator of GlgC [36]. It is thus expected that mutants impaired in the synthesis of this purine nucleotide will display AMP-deficient and glycogen-excess phenotypes. Consistent with this presumption, mutants of genes that code for enzymes involved in the *de novo* synthesis of AMP such as *ΔpurA*, *ΔpurC*, *ΔpurD*, *ΔpurE*, *ΔpurF*, *ΔpurH*, *ΔpurL*, and *ΔpurM* displayed glycogen-excess phenotypes and accumulated low AMP levels when compared to WT cells (**Figure 4** and **Figure 8**, respectively). Furthermore, cells ectopically expressing *purB* displayed AMP-excess and glycogen-deficient phenotypes (**Figure 9**).

### End-turnover of tRNA

End-turnover of tRNA consists of the removal and readdition of the 3' terminal AMP residues to uncharged tRNA. RNase T (the product of *mnt*) is a nuclease highly specific for uncharged tRNA-C-C-A that releases AMP and tRNA-C-C, and that highly controls tRNA turnover in *E. coli* [73]. Cells impaired in tRNA turnover accumulate defective tRNA molecules, leading to arrest of protein biosynthesis as a consequence of elevation of ppGpp levels [74]. In agreement with the idea that the stringent response plays a pivotal role in glycogen metabolism, *Δmnt* mutants displayed a glycogen excess phenotype (**Figure 4**).

### Additional remarks

Results presented in this work show that glycogen metabolism is a highly regulated process that is connected with a wide variety of cellular functions. In addition, we show that extracellular  $Mg^{2+}$  is an important determinant of both energy status of the cell and glycogen accumulation. Noteworthy, analyses of the intracellular AMP levels in mutants representing all categories of both glycogen-excess and glycogen-deficient mutants revealed a trend-wise reverse correlation between AMP and glycogen contents (**Figure 8**). No such correlation could be found between ADP and glycogen content (**Supplemental Figure 5**). This observation is consistent with the view discussed above that AMP plays a major role in regulating glycogen metabolism in *E. coli*. To further explore this hypothesis we carried out a time-course analysis of AMP and glycogen contents in cells cultured in liquid glucose Kornberg medium. As shown in **Figure 10**, these analyses clearly revealed a reverse correlation between intracellular glycogen and AMP contents, the overall data further supporting the idea that AMP acts a major determinant of glycogen metabolism in *E. coli*.

**Figure 11** illustrates a suggested model of glycogen metabolism wherein major determinants of glycogen accumulation include levels of intracellular Mg-bound ATP necessary for GlgC activity (strongly determined by extracellular  $Mg^{2+}$  concentrations, transport of  $Mg^{2+}$  across membranes, ATP synthesis and consumption, and possibly Adk activity), levels of AMP (the main GlgC inhibitor), levels of ppGpp (which

accumulates under conditions of limited provision of nutrients such as amino acids, sulfur,  $Mg^{2+}$ , iron, etc.), levels of cAMP, availability of a carbon source, redox status of the cell, and less well-defined systems sensing the cell energy status through the activity of the electron transport chain. According to this model, under conditions of limited nutrient provision, a decreased demand in ATP-dependent protein and nucleic acid synthesis will take place, and excess ATP will be diverted towards glycogen biosynthesis when an excess carbon source is present in the medium. Adk activity represents an important tool for maintaining  $Mg^{2+}$  homeostasis and generating the membrane potential that drives nutrient transport and ATP synthesis. It allows amplification of extracellular  $Mg^{2+}$ -dependent small changes in ATP and ADP concentrations into proportionally larger changes in AMP concentrations.

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## Legends to figures

**Figure 1: Extracellular  $Mg^{2+}$  in the submillimolar range is an important determinant of intracellular glycogen content in  $\Delta mgtA$ ,  $\Delta phoP$  and  $\Delta phoQ$  cells.**

(A) Glycogen content at the onset of the stationary phase in  $\Delta mgtA$ ,  $\Delta phoP$ ,  $\Delta phoQ$  and WT cells cultured in glucose Kornberg liquid medium with the indicated  $Mg^{2+}$  concentrations. (B) Iodine staining of WT and  $\Delta mgtA$ ,  $\Delta phoP$ ,  $\Delta phoQ$  cells cultured in glucose Kornberg solid medium containing the indicated  $Mg^{2+}$  concentrations.

**Figure 2: Extracellular  $Mg^{2+}$  is an important determinant of *E. coli* intracellular ATP levels.** ATP contents were determined at the onset of the stationary phase in WT cells cultured in glucose Kornberg liquid medium containing the indicated  $Mg^{2+}$  concentrations. The mean  $\pm$  SE of 4 independent experiments are shown. For details see Materials and Methods.

**Figure 3: *glgCAP* is under positive control of the PhoP-PhoQ regulatory system in *E. coli* under limiting  $Mg^{2+}$  conditions.** Comparative analysis of growth (A,C) and expression of chromosomal *glgC::lacZY* fusions (B,D) between WT cells (■),  $\Delta phoP$  (●),  $\Delta phoQ$  (◆) and  $\Delta mgtA$  (▲) cells cultured in glucose Kornberg liquid medium either under limiting (A,B) and non-limiting (C,D)  $Mg^{2+}$  conditions (50  $\mu M$  and 1 mM  $Mg^{2+}$ , respectively). (E) Western blot analysis of GlgC in WT cells (lanes 1 and 2),  $\Delta phoP$  mutants (lanes 3 and 4)  $\Delta phoQ$  mutants (lanes 5 and 6) and *phoQ* expressing  $\Delta phoQ$  cells (lane 7) cultured in glucose Kornberg liquid medium under limiting (lanes 1, 3, 5 and 7) and non-limiting (lanes 2, 4 and 6)  $Mg^{2+}$  conditions (50  $\mu M$  and 1 mM  $Mg^{2+}$ , respectively). The lower panel shows the Coomassie blue stained gel in the polypeptide region 40-55 kDa corresponding to GlgC migration. The gel was loaded with 30  $\mu g$  per lane of total soluble proteins of each of the described cells. For details see Materials and Methods.

**Figure 4: Intracellular glycogen content of glycogen-excess mutants of the Keio collection.** Glycogen content is referred as percentage of glycogen accumulated by WT cells. Average glycogen content in WT cells was 140 nmol glucose/mg protein. Cells were cultured in liquid glucose Kornberg medium containing 250  $\mu M$   $Mg^{2+}$  and harvested at the onset of the stationary phase.

**Figure 5: Intracellular glycogen content of both glycogen-less and glycogen-deficient mutants of the Keio collection.** Glycogen content is referred as percentage of glycogen accumulated by WT cells. Average glycogen content in WT cells was 140 nmol glucose/mg protein. Cells were cultured in liquid glucose Kornberg medium containing 250  $\mu M$   $Mg^{2+}$  and harvested at the onset of the stationary phase.

**Figure 6: Functional categorization of the genes whose expression affects glycogen content in *E. coli*.** Genes whose expression leads to glycogen-excess phenotype are in black bars, whereas genes whose expression leads to glycogen-deficient phenotype are in white bars.

**Figure 7: The *E. coli glgCAP* operon is under control of RelA.** Comparative analysis of (A) growth and (B) expression of *glgC::lacZY* fusions in WT cells (\*),  $\Delta cya$  (◆),

*Δfur* (●), *ΔrpoS* (■) and *ΔrelA* cells (▲) cultured in glucose Kornberg liquid medium containing 250 μM Mg<sup>2+</sup>.

**Figure 8: AMP content in mutants representing the different glycogen-excess and glycogen-deficient categories of the Keio collection.** Average AMP content in WT cells was 0.95 nmol AMP/mg protein. Cells were cultured in glucose Kornberg medium containing 250 μM Mg<sup>2+</sup> and harvested when entering the stationary phase. White bars correspond to glycogen-less or glycogen-deficient mutants, whereas black bars correspond to glycogen-excess mutants.

**Figure 9: Intracellular AMP is a major determinant of glycogen accumulation.** (A) Iodine staining, (B) glycogen content and (C) AMP content in WT and *purB* over-expressing cells cultured in solid glucose Kornberg medium containing 250 μM Mg<sup>2+</sup>. The results are the mean ± SE of 4 independent experiments.

**Figure 10: Time-course analyses of AMP and glycogen content in *E. coli*.** Time-course analyses of (A) culture growth, (B) AMP content and (C) glycogen content in WT cells cultured in liquid cultures of glucose Kornberg medium containing 250 μM Mg<sup>2+</sup>.

**Figure 11: Integrated model of glycogen metabolism.** Suggested metabolic model wherein major determinants of glycogen metabolism include the PhoP-PhoQ regulatory system activated under low extracellular Mg<sup>2+</sup> concentration conditions, intracellular concentration of Mg-bound ATP necessary for GlgC activity (determined by extracellular Mg<sup>2+</sup> concentration, transport of Mg<sup>2+</sup> across membranes, ATP synthesis and consumption, and Adk activity), levels of AMP (the main GlgC inhibitor), levels of ppGpp (which accumulates under conditions of limited provision of nutrients such as amino acids, sulfur, iron, etc.), levels of cAMP, availability of a carbon source, redox status of the cell, and less well-defined systems sensing the cell energy status through the activity of the electron transport chain. According to this model, under conditions of limited nutrient provision, a decreased demand in ATP-dependent protein and nucleic acid synthesis will take place, and excess ATP will be used for glycogen biosynthesis when glucose is present in the medium.

**Table 1. *E. coli* genes whose deletions caused a “glycogen-excess” phenotype.** Genes are classified into COG categories [24,75,76]. The numbers in parentheses represent the number of glycogen-related genes to the number of genes belonging to each of COG category.

### Metabolism

- C. Energy production and conversion (7/311)  
*aceA, atpA, paaA, paaD, paaE, pntA, pntB*
- E. Amino acid transport and metabolism (19/446)  
*argH, aroA, aroC, aroF, aroK, carA, carB, cysC, cysE, cysH, cysM, glnB, metF, proW, serA, serB, serC, tyrA, ygaZ*
- F. Nucleotide transport and metabolism (11/92)  
*cmk, purA, purC, purD, purE, purF, purH, purL, purM, pyrB, pyrE*
- G. Carbohydrate transport and metabolism (6/426)  
*glgP, glgX, rbsA, rbsB, ybhE, zwf*
- H. Coenzyme transport and metabolism (1/152)  
*cysG*
- P. Inorganic ion transport and metabolism (13/284)  
*cysA, cysD, cysI, cysJ, cysN, cysP, cysU, cysW, fepB, fepD, fepG, phoU, yebZ*

### Cellular processes

- O. Posttranslational modification, protein turnover, chaperones (8/140)  
*clpA, clpP, dnaK, dsbA, dsbB, glnD, lon, ydhD*
- U. Intracellular trafficking, secretion, and vesicular transport (1/136)  
*tatB,*

### Information, storage and processing

- J. Translation, ribosomal structure and biogenesis (2/182)  
*pnp, yciO,*
- K. Transcription (3/329)  
*deoT, uidR, ydeO,*
- L. DNA replication, recombination and repair (5/236)  
*dnaQ, nudB, parC, rnt, ybaZ*

### Poorly characterized

- R. General function prediction only (6/517)  
*hflD, mdtH, yahD, ybgC, yobA, yfbW*
- S. Function unknown (6/326)  
*ybgI, yciS, yjcH, ynfA, yeaC, yeeX,*

### No COG assignment (6/432)

*crl, pyrL, yncH, yheO, yqiI, yqiJ*



**Table 2. *E. coli* genes whose deletions caused a “glycogen-deficient” or “glycogen-less” phenotype.** Genes are classified into COG categories [24,75,76]. The numbers in parentheses represent the number of glycogen-related genes to the number of genes belonging to each of COG category.

### Metabolism

- C. Energy production and conversion (7/311)  
*fdx, gor, iscU, lpd, nuoF, ubiH, ygiQ*
- E. Aminoacid transport and metabolism (5/446)  
*allC, aroB, aroE, iscS, ydgI*
- F. Nucleotide transport and metabolism (4/92)  
*cyaA, pyrC, pyrF, yjjY*
- G. Carbohydrate transport and metabolism (10/426)  
*galU, glgA, glgB, glgC, treC, pgi, pgm, ptsG, ptsH, ptsI*
- H. Coenzyme transport and metabolism (2/152)  
*gshB, ubiG*
- I. Lipid transport and metabolism (2/108)  
*paaH, yfjG*
- P. Inorganic ion transport and metabolism (11/284)  
*cysQ, fur, kdpB, pitA, pstA, pstB, pstC, yccK, yheL, yheM, yheN*

### Cellular processes

- D. Cell cycle control, cell division, chromosome partitioning (1/36)  
*yafN*
- M. Cell wall/membrane/envelope biogenesis (5/246)  
*mrcB, rfaE, tolB, tolQ, tolR*
- O. Posttranslational modification, protein turnover, chaperones (2/140)  
*hscB, yhhP*
- T. Signal transduction mechanisms (6/210)  
*arcA, citA, crp, dksA, relA, rseA*
- V. Defense mechanisms (1/50)  
*ldcA*

### Information, storage and processing

- J. Translation, ribosomal structure and biogenesis (8/182)  
*dusB, prfC, rluD, rpmF, rpmJ, rrmJ, srmB, trmU*
- K. Transcription (7/329)  
*fis, gcvA, greA, nikR, nusB, rpoN, rpoS*
- L. DNA replication, recombination and repair (5/236)  
*holD, ihfA, ihfB, ruvA, ruvB*

### Poorly characterized

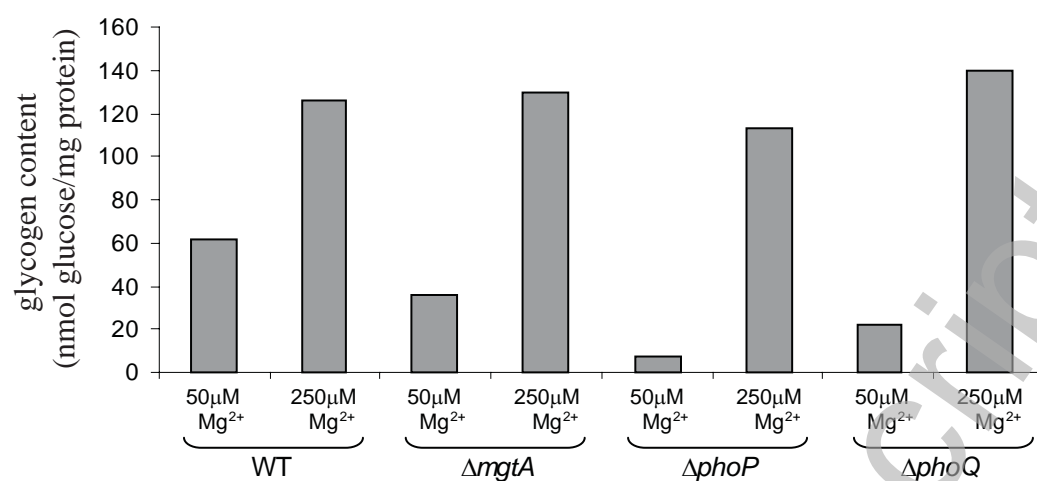
- R. General function prediction only (8/517)  
*hfg, , rsgA, trmE, yciQ, yddG, ydfO, ydhF, yfbJ*
- S. Function unknown (4/326)  
*ydcQ, yggT, yhbC, yiiS*

### No COG assignment (3/432)

*glgS, yhdN, yneK*



A



B

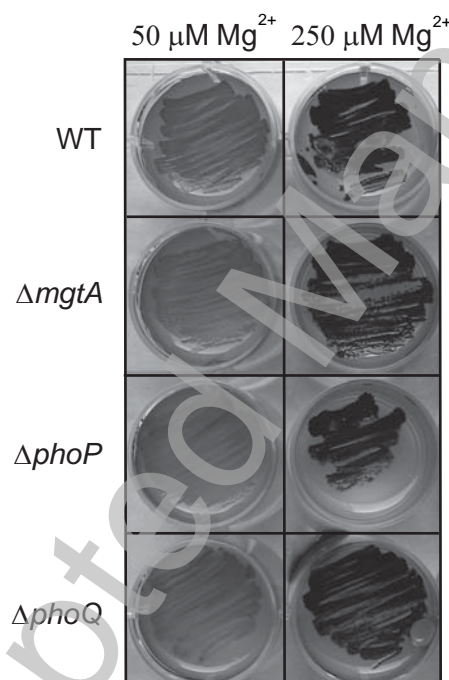


Figure 1

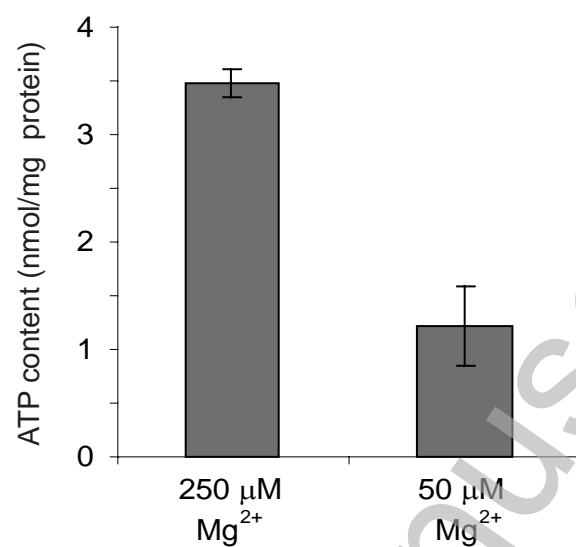


Figure 2

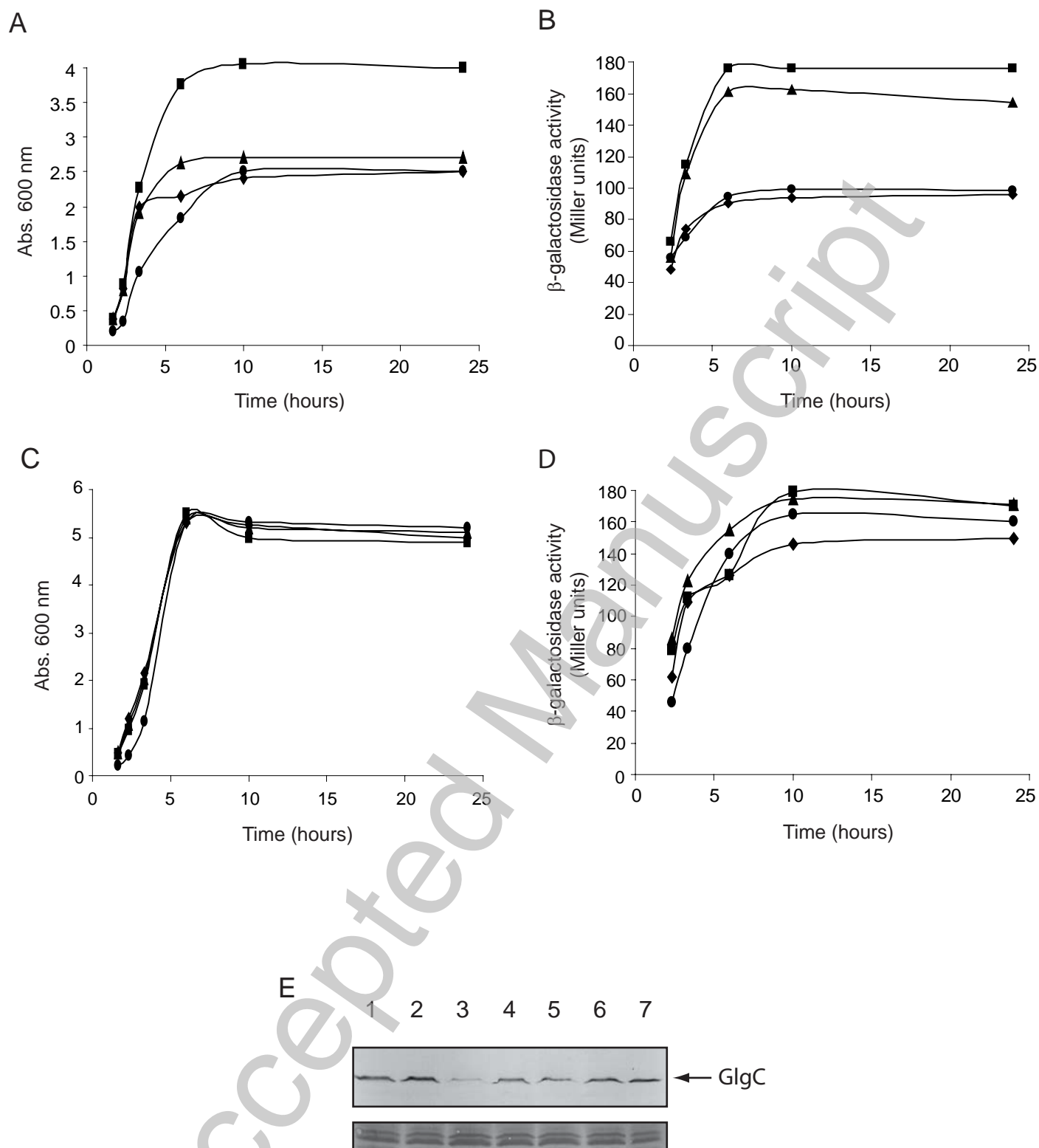


Figure 3

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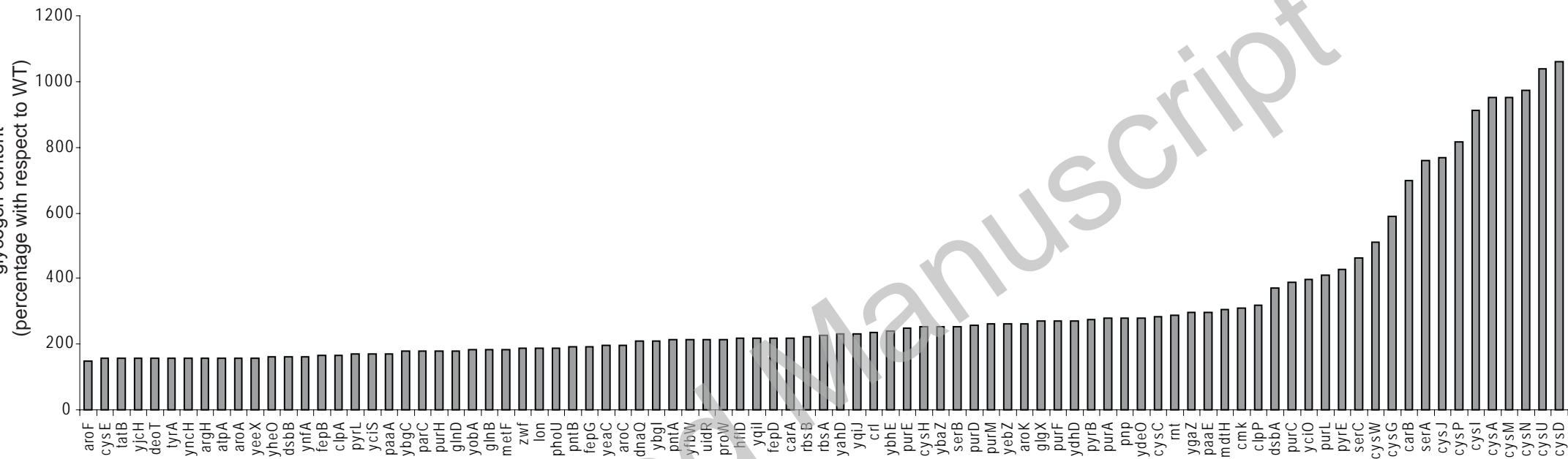


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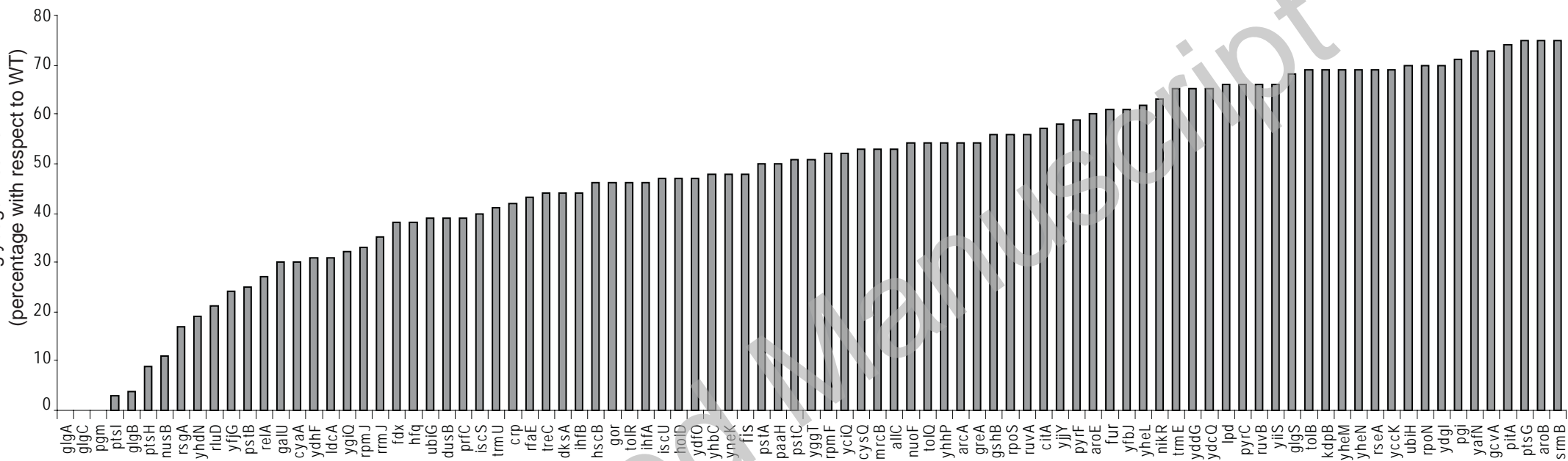


Figure 5

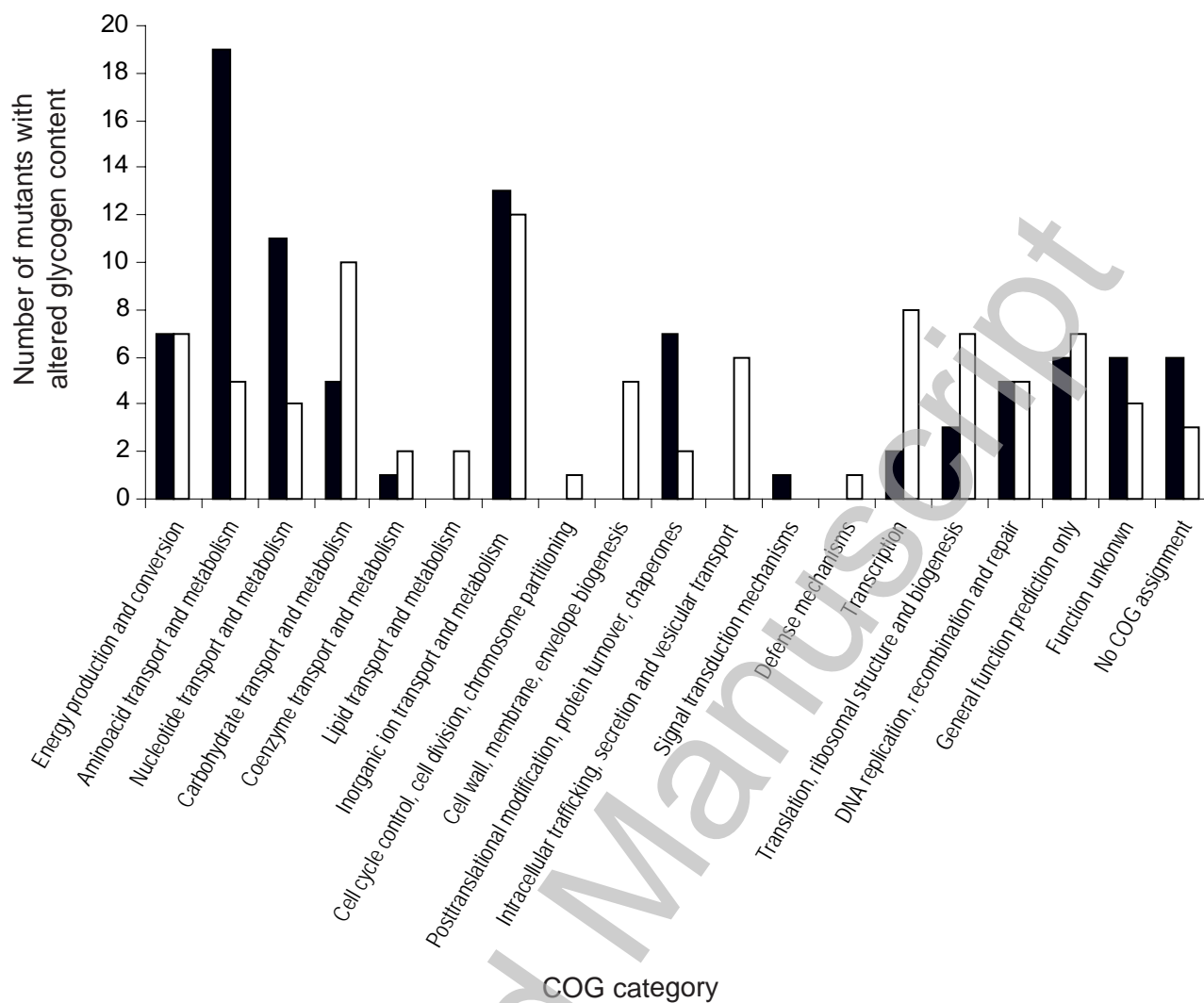
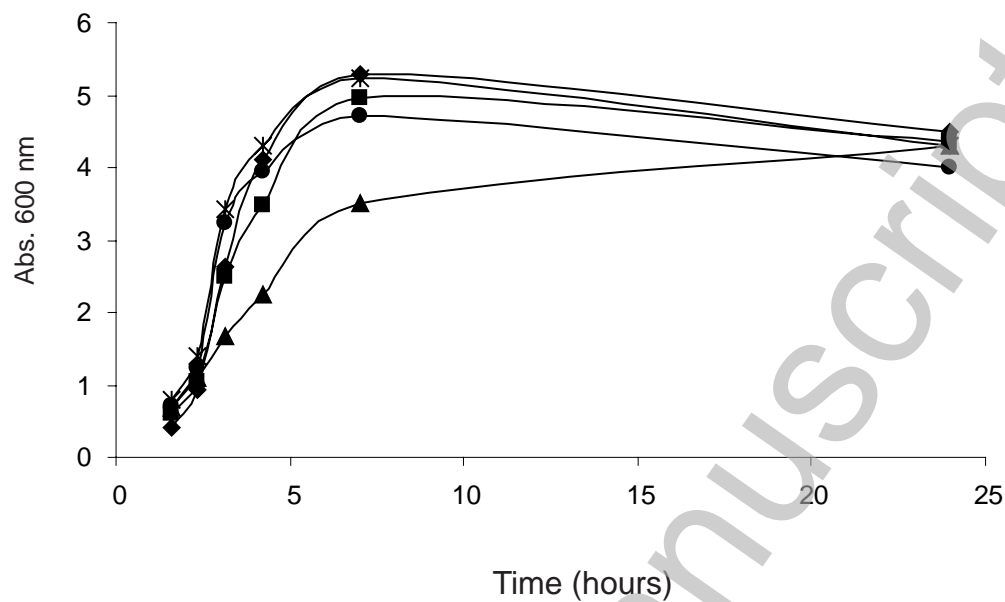


Figure 6



A



B

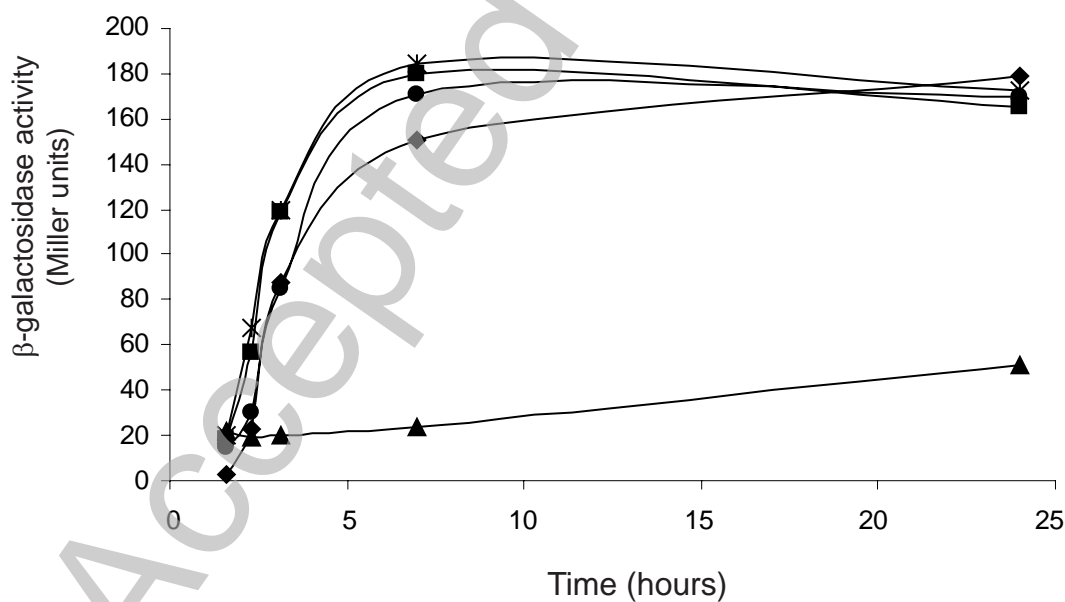


Figure 7

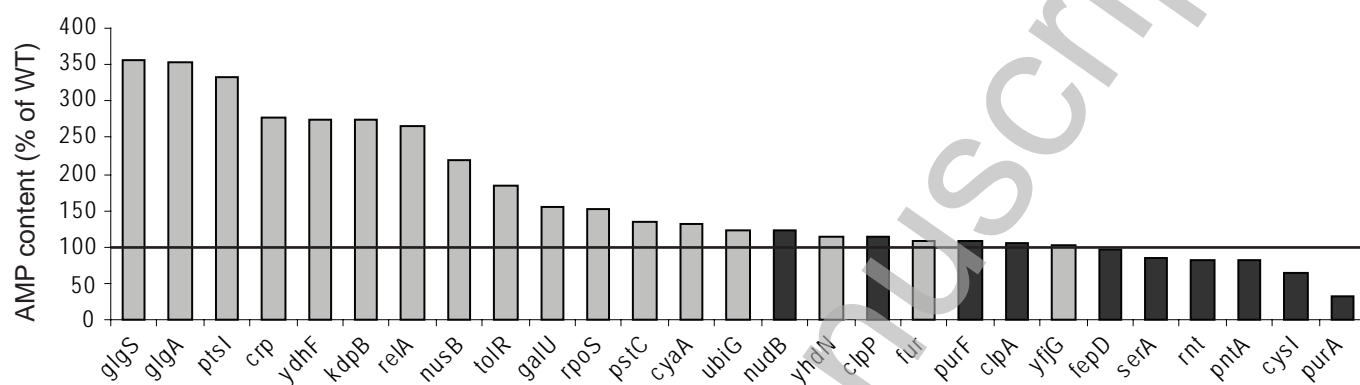


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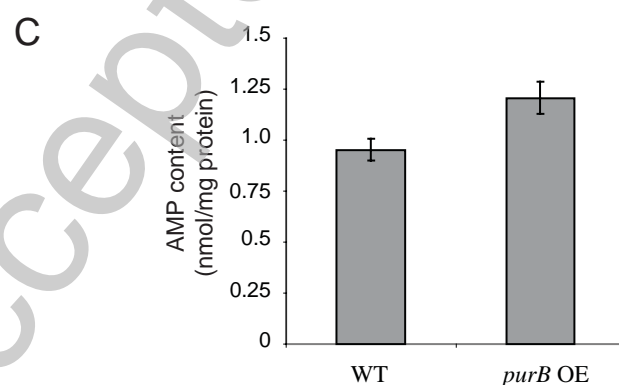
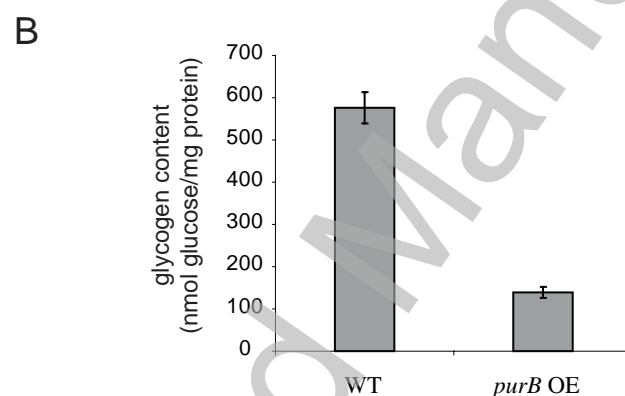
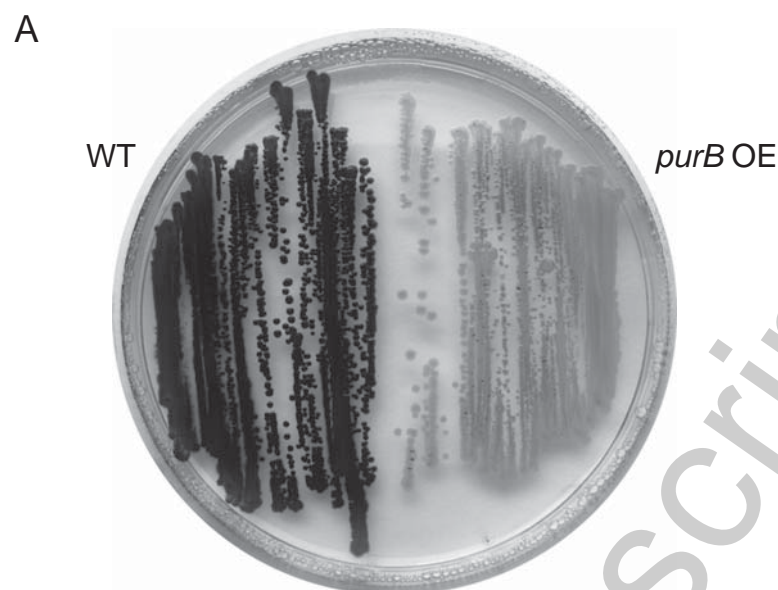


Figure 9

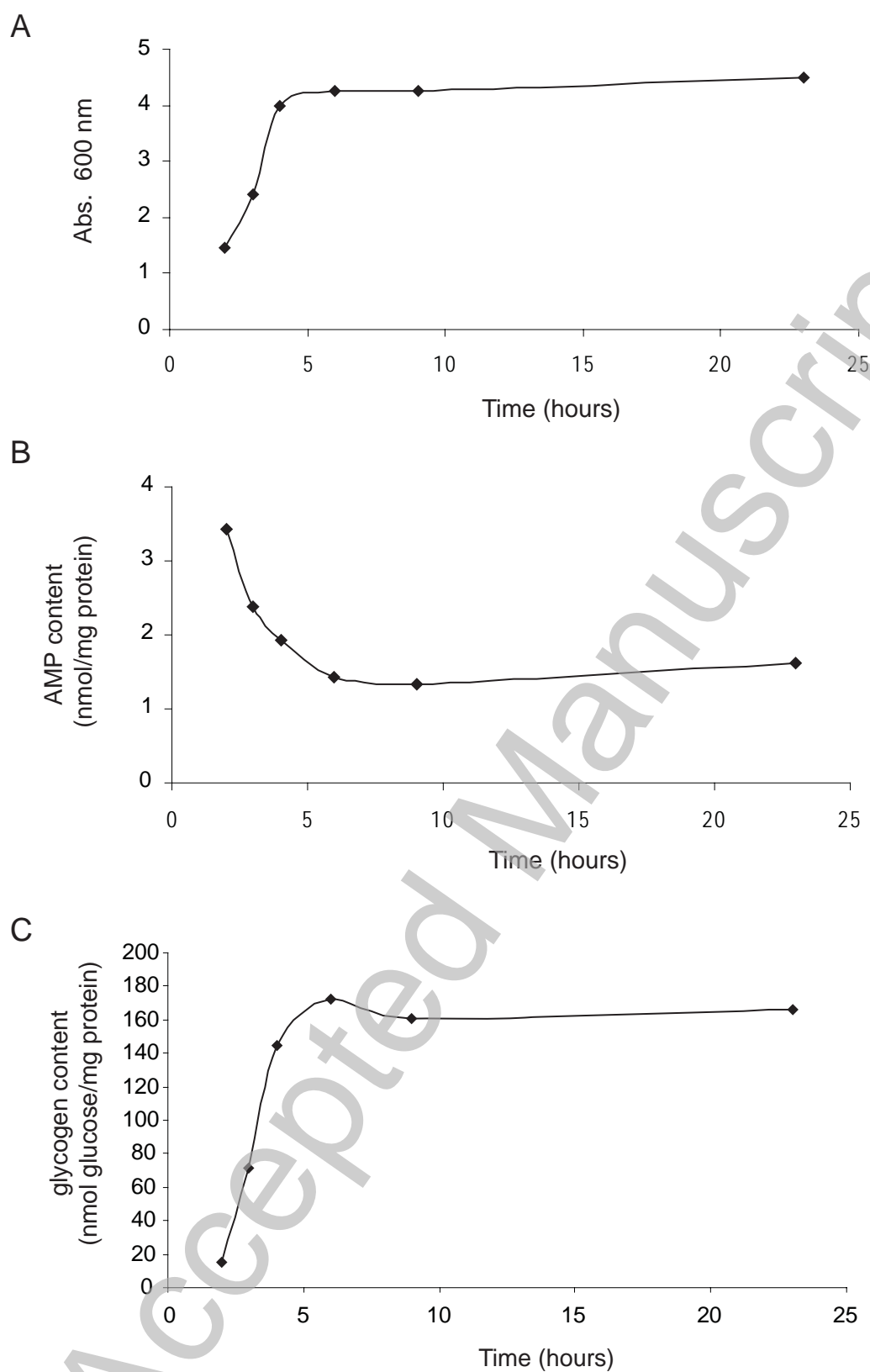


Figure 10

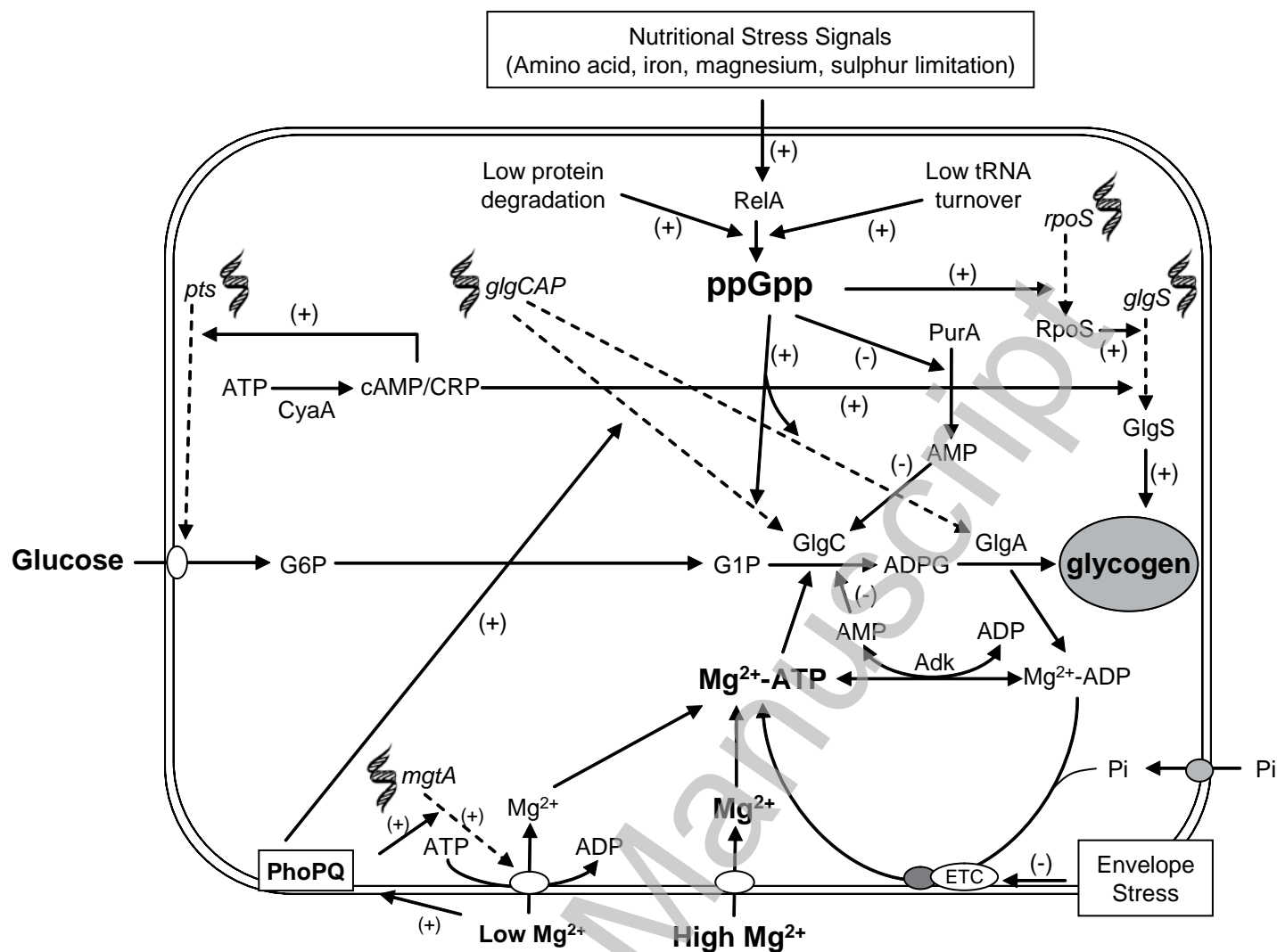


Figure 11