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Genome-Wide Screening of Genes Whose Enhanced Expression Affects Glycogen Accumulation in *Escherichia coli*

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Abstract

Using a systematic and comprehensive gene expression library (the ASKA library), we have carried out a genome-wide screening of the genes whose increased plasmid-directed expression affected glycogen metabolism in *Escherichia coli*. Of the 4123 clones of the collection, 28 displayed a glycogen-excess phenotype, whereas 58 displayed a glycogen-deficient phenotype. The genes whose enhanced expression affected glycogen accumulation were classified into various functional categories including carbon sensing, transport and metabolism, general stress and stringent responses, factors determining intercellular communication, aggregative and social behaviour, nitrogen metabolism and energy status. Noteworthy, one-third of them were genes about which little or nothing is known. We propose an integrated metabolic model wherein *E. coli* glycogen metabolism is highly interconnected with a wide variety of cellular processes and is tightly adjusted to the nutritional and energetic status of the cell. Furthermore, we provide clues about possible biological roles of genes of still unknown functions.

Key words: ASKA library; carbohydrate metabolism; functional genomics

1. Introduction

Glycogen is a major intracellular reserve polymer consisting of α-1,4-linked glucose subunits with α-1,6-linked glucose at the branching points, which accumulates in *Escherichia coli* and other bacteria under conditions of limiting growth when an excess of carbon source is available and other nutrients are deficient.[1–3] The exact role of this polyglucan in bacteria is still not well-defined, but several works have linked glycogen metabolism to environmental survival, symbiotic performance and colonization and virulence.[4–12]

Bacterial glycogen is produced by the concerted action of glycogen synthase (GlgA) and branching enzyme (GlgB) using ADP-glucose (ADPG) as the sugar donor nucleotide.[1] Since the initial demonstration that ADPG serves as the precursor molecule for bacterial glycogen biosynthesis,[13] it has been considered that ADPG pyrophosphorylase (GlgC) is the sole enzyme catalyzing the production of ADPG in these organisms.[14] However, recent reports have provided evidence about the occurrence of other important sources of ADPG linked to glycogen biosynthesis in bacterial species such as *E. coli*, *Salmonella*, *Streptomyces coelicolor* and *Mycobacterium tuberculosis*.[11,15–17] Genes involved in glycogen metabolism in enterobacterial species, such as *E. coli* and *Salmonella enterica*, are clustered in two apparently independent transcriptional units designated as *glgBX* (encoding GlgB and debranching GlgX enzymes) and *glgCAP* [comprising genes coding for

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the glycogen anabolic enzymes GlgC and GlgA, and the catabolic glycogen phosphorylase (GlgP)].[1]

Regulation of *E. coli* glycogen metabolism involves a complex assemblage of factors that are adjusted to the physiological and energetic status of the cell,[2,3,18,19] and cell-to-cell communication.[20] At the level of enzyme activity, glycogen metabolism is subjected to the allosteric regulation of GlgC by different glycolitic intermediates.[14] Also, *E. coli* GlgP activity is regulated by the phosphorylation status of the carbohydrate phosphotransferase system (PTS) protein Hpr.[21] At the level of gene expression, several factors have been described to control *E. coli* glycogen accumulation. This includes negative regulation by the carbon storage regulator CsrA and by the still unidentified *glgQ* regulatory locus,[22–24] and positive regulation by guanosine 5′-triphosphate 3′-diphosphate and/or guanosine 5′-diphosphate 3′-diphosphate [(p)ppGpp] stringent response regulators[3,25–28] and by the PhoP–PhoQ regulatory system at low environmental Mg^{2+} concentration.[3] Different experimental evidences also indicate positive regulation of *glgCAP* expression by the cyclic AMP/cyclic AMP receptor protein complex[29–31] (however, for an opposite view, see Montero *et al*.[3] and Hengge-Aronis and Fischer[32]). The general stress regulator RpoS does not regulate *glgCAP* expression, but positively controls the expression of *glgS*, a gene whose product exerts a positive effect on glycogen accumulation.[32]

We have recently initiated a series of studies aimed to uncover mechanisms regulating bacterial glycogen metabolism and its connection with other biological processes. Using a systematic and comprehensive gene-disrupted mutant collection of *E. coli* (the Keio collection[33]), we carried out genome-wide screenings of genes affecting glycogen metabolism in this bacterial species.[2,3] Our studies revealed that bacterial glycogen metabolism is highly interconnected with a wide variety of cellular processes and proposed an integrated metabolic model wherein glycogen metabolism is influenced by the stringent and general stress responses, end-turnover of tRNA, intracellular AMP levels, nutrient transport and metabolism, low extracellular Mg^{2+} availability and energy production.[3] To further investigate the mechanisms regulating bacterial glycogen metabolism and its connection with other biological processes, in this work, we have carried out a genome-wide analysis of glycogen content using the ASKA library, a set of 4123 clones expressing all predicted ORFs of an *E. coli* K-12 derivative.[34] The overall data presented in this work reinforce the idea that glycogen metabolism is highly interconnected with a wide variety of cellular processes and is adjusted to the bacterial energy and nutritional status. Furthermore, we provide evidence showing that glycogen metabolism is also affected by factors determining intercellular communication, aggregative and social behaviour modes.

2. Materials and methods

2.1. Bacterial strains and culture conditions

We used the ASKA library, a set of 4123 different clones of the AG1 *E. coli* K-12 strain (*recA1 endA1 gyrA96 thi-1 hsdR17(rK_mKþ) supE44 relA1*), each expressing one of all predicted *E. coli* K-12 ORFs.[34] For quantitative measurement of glycogen content, cells were grown at 37°C with rapid gyratory shaking in liquid Kornberg medium (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract from Duchefa, Haarlem, the Netherlands) supplemented with 50 mM glucose and 1 mM $MgCl_2$ after inoculation with 1 vol. of an overnight culture per 50 volumes of fresh medium. The culture medium was not supplemented with IPTG. Cultures entering the stationary phase were centrifuged at 4400*g* for 15 min, and the collected cells were rinsed with fresh Kornberg medium, resuspended in 40 mM Tris–HCl (pH 7.5) and disrupted by sonication as described previously.[2] Solid culture medium was prepared by adding 1.8% bacteriological agar to liquid Kornberg medium before autoclaving.

2.2. Screening of ASKA clones with altered glycogen content

A first screening of glycogen in the different bacterial clones of the ASKA collection after growth on solid glucose Kornberg medium was carried out employing the iodine staining method. In the presence of iodine vapours, 'glycogen-excess' clones stained darker than its brownish parent cells, whereas 'glycogen-deficient' clones stained yellow.[16] Clones identified using this procedure were subsequently cultured in liquid glucose Kornberg medium and subjected to the quantitative measurement of glycogen content at the onset of the stationary phase 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 (USA) prepared reagent. The function of each gene whose enhanced expression affects glycogen accumulation was assigned by referring to the EchoBASE (http://ecoli-york.org/)[35] and EcoCyc (http://www.ecocyc.org/)[36] databases.

2.3. Morphotype evaluation

To monitor the expression of curli and cellulose biosynthesis, 10 μl of a bacterial overnight culture suspended in water to an absorbance at 600 nm of five were spotted onto TY agar plates (1% Bacto

Tryptone, 0.5% yeast extract, 1.5% bacteriological agar) supplemented with 40 μg ml^{-1} Congo red and 20 μg ml^{-1} Coomassie brilliant blue.[37] Plates were incubated at 28°C for 5 days, and dye binding was evaluated by red colour intensity. The multicellular rdar morphotype is characterized by a red, dry and rough aspect on Congo red agar plates, which is determined by the expression of extracellular matrix components such as cellulose and adhesive curli fimbriae.[38] The appearance of a pink colony (pdar morphotype) is indicative of cellulose biosynthesis.[39] Capacity for cellulose production was also qualitatively analyzed by assessing the level of calcofluor white (Fluorescent brightener 28; Sigma) binding of colonies grown on TY agar plates supplemented with 50 μg ml^{-1} of this dye. Fluorescence of the cells was observed under a 366 nm UV light source and compared with the wild-type (WT) strain.

2.4. *General molecular techniques*

Routine DNA manipulations were performed following standard procedures.[40] Plasmids were extracted by Quantum Prep plasmid mini-prep kit (Bio-Rad). Δ*relA* cells of the Keio collection[33] expressing *relA* in *trans* were obtained by incorporation of *relA*- expression vector of the ASKA library. DNA sequencing was carried out in Secugen (Madrid). Sequence homologies to genes in the GenBank database were determined by using the BLAST algorithm of the National Center for Biotechnology Information at the National Library of Medicine.

2.5. *Analytical procedures*

Bacterial growth was followed spectrophotometrically by measuring the absorbance of cultures at 600 nm. Protein contents in bacterial extracts were measured by the Coomassie G dye-binding method using a Bio-Rad prepared reagent.

3. Results and discussion

3.1. *Screening, identification and classification of genes whose enhanced expression affects glycogen accumulation*

Clones of ASKA collection were first screened for altered glycogen content in solid glucose Kornberg medium. In the presence of iodine vapours, 'glycogen-excess' clones stained darker than their brownish parent cells, whereas 'glycogen-deficient' clones stained yellow. On inspecting the ASKA library, 28 clones (0.7% of the library) displayed 'glycogen-excess' phenotypes, whereas 58 clones (1.4% of the library) displayed yellow, 'glycogen-deficient' phenotypes. Subsequent quantitative glycogen measurement analyses on cells entering the stationary phase confirmed that the 86 selected clones accumulate altered levels of glycogen (Fig. 1).

The 86 genes whose enhanced expression showed modified glycogen accumulation were classified into clusters of orthologous groups (COGs).[41] Tables 1 and 2 show the genes whose enhanced expression leads to glycogen-excess and glycogen-deficient phenotypes, respectively, whereas Supplementary Table S1 shows the function of each gene product. In some cases, the families are clearly meaningful, with the presence of multiple genes of related function, reinforcing the validity of their identification in the survey. Yet, a large group of 28 clones, representing one-third of the clones identified, express genes about which little or nothing is known such as *glgS*, *gspD*, *mdtG*, *ppdB*, *rutF*, *smg*, *ucpA*, *yabI*, *yafV*, *ybcV*, *ycbJ*, *yciN*, *ydcJ*, *yegH*, *yfaY*, *yfdN*, *yfeD*, *yfjR*, *yhcE*, *yjcC*, *yjcQ*, *ylcG*, *ymgC*, *ynbD*, *yncC*, *yncG*, *yoaE* and *yqjA*.

The general trend observed after this analysis indicates that glycogen metabolism of *E. coli* cells cultured in glucose Kornberg medium is affected by genes whose products can be embodied in the following groups:

- Carbon sensing, transport and metabolism;
- general stress response;
- stringent response;
- factors determining intercellular communication, aggregative and social behaviour;
- nitrogen metabolism;
- energy status.

3.1.1. *Carbon sensing, transport and metabolism*

As expected from the glycogen synthetic roles of GlgA and GlgC, *glgA* and *glgC* over-expressing bacteria of the ASKA library displayed glycogen-excess phenotypes (Fig. 1). In fact, these bacteria presented the highest levels of glycogen accumulation of the whole collection. In agreement also with the assigned function of GlgP and AspP in *E. coli* glycogen breakdown,[42,43] AG1 cells over-expressing *glgP* and *aspP* showed reduced glycogen accumulation (Fig. 1). Noteworthy, although GlgB is a glycogen anabolic enzyme, *glgB* over-expressing cells of the ASKA library displayed a glycogen-deficient phenotype (Fig. 1). This could be ascribed to the fact that glycogen granule architecture is the result of the highly orchestrated actions of GlgB and other glycogen enzymes, which may collapse under GlgB over-production conditions.[44]

The global regulator of carbon metabolism CsrA is an RNA-binding protein, which is thought to prevent glycogen biosynthesis by both promoting *glgCAP* decay and translation.[24,45] Consistently, *csrA* over-expressing bacteria of the ASKA library displayed a glycogen-deficient phenotype (Fig. 1). CsrA activity is

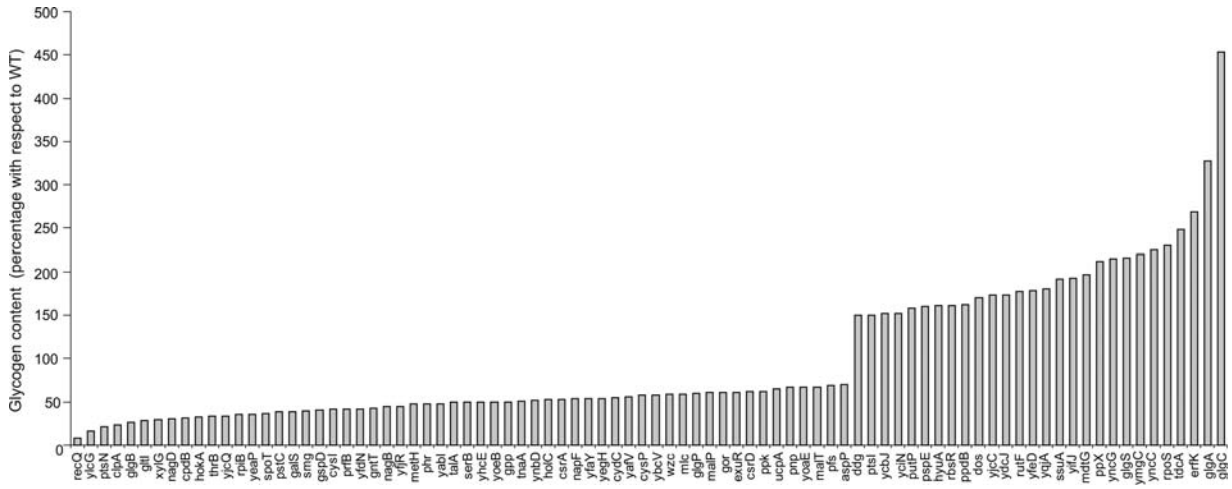

Figure 1. Glycogen content (referred as percentage of glycogen accumulated by WT cells) of glycogen-excess and glycogen-deficient clones of the ASKA library. Averaged glycogen content in WT cells was 45 nmol glucose mg protein^{-1}. Cells were grown at 37°C with rapid gyratory shaking in liquid Kornberg medium (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract) supplemented with 50 mM glucose. Because some yeast extracts are deficient in Mg^{2+},[3] and because Mg^{2+} is a major determinant of cell metabolic and energetic status and of expression of genes affecting glycogen metabolism,[91,92] the Kornberg medium was also supplemented with 1 mM $MgCl_2$.

Table 1. *Escherichia coli* genes whose enhanced expression caused a 'glycogen-excess' phenotype in cells of the ASKA library entering the stationary phase

Metabolism	
E	Amino acid transport and metabolism (1/432): *putP*
F	Nucleotide transport and metabolism (1/94): *hyuA*
G	Carbohydrate transport and metabolism (3/395): *glgA*, *glgC*, *ptsI*
P	Inorganic ion transport and metabolism (3/273): *ppx*, *pspE*, *ssuA*
Cellular processes	
M	Cell wall/membrane/envelope biogenesis (1/227): *ddg*
O	Posttranslational modification, protein turnover, chaperones (1/144): *yncG*
U	Intracellular trafficking (1/116): *ppdB*
Information, storage and processing	
K	Transcription (5/321): *rbsR*, *rpoS*, *tdcA*, *yfeD*, *yncC*
T	Signal Transduction (2/186): *dos*, *yjcC*
Poorly characterized	
R	General function prediction only (3/510): *mdtG*, *rutF*, *yifJ*
S	Function unknown (3/315): *erfK*, *ydcJ*, *yqjA*
	No COG assignment (4/590): *glgS*, *ycbJ*, *yciN*, *ymgC*

Genes are classified into COG categories.[35,36,41] The numbers in parentheses represent the number of glycogen-related genes to the number of genes belonging to each of COG category.

antagonized by the two CsrB and CsrC non-coding RNAs,[46–48] which in turn are targeted by CsrD for RNase E degradation.[49] Thus and consistent with the assigned role of CsrD as relieving CsrA function from CsrB and CsrC, *csrD* over-expressing cells of the ASKA library displayed a glycogen-deficient phenotype (Fig. 1).

malP and *malT* over-expressing bacteria of the ASKA collection displayed glycogen-deficient phenotypes (Fig. 1). MalT is a transcriptional regulator of genes involved in maltose/maltodextrin transport and metabolism.[50,51] MalP, over which MalT exerts a positive control, catalyzes the phosphorolytic breakdown of maltodextrins. However, MalP poorly recognizes large and highly branched polyglucans such as glycogen,[42] suggesting an indirect rather than a direct effect of *malP* over-expression on glycogen accumulation. In this respect, previous studies have indicated tight, albeit still not well characterized, links between glycogen and maltodextrin metabolisms.[52,53]

PTS is a major determinant of transport and phosphorylation of a large number of carbohydrates including glucose.[21,54,55] PTS mutants impaired in sensing and transport of glucose accumulate low glycogen content.[3] It is therefore conceivable that over-expression of some PTS components would result in enhanced glycogen content in cells cultured in glucose Kornberg medium, whereas cells over-expressing the Mlc transcriptional repressor of PTS genes[56] would display a glycogen-deficient phenotype. Confirming this presumptions *ptsI* over-expressing bacteria of the ASKA library displayed a glycogen-excess phenotype (Fig. 1), whereas cells ectopically expressing the Mlc transcriptional repressor of PTS genes displayed a glycogen-deficient phenotype (Fig. 1).

3.1.2. General stress response Different genetic studies indicate a requirement of the general stress regulator RpoS as a positive modulator of glycogen biosynthesis.[2,3,57] In agreement, *rpoS* over-expressing

Table 2. *Escherichia coli* genes whose enhanced expression caused a 'glycogen-deficient' phenotype

Metabolism	
C	Energy production and conversion (2/301): *gor*, *napF*
E	Amino acid transport and metabolism (5/432): *gltI*, *metH*, *serB*, *thrB*, *tnaA*
F	Nucleotide transport and metabolism (3/94): *cpdB*, *gpp*, *pfs*
G	Carbohydrate transport and metabolism (10/395): *glgB*, *glgP*, *gntT*, *malP*, *nagB*, *nagD*, *ptsN*, *rpiB*, *talA*, *xylG*
I	Lipid transport and metabolism (1/104): *ynbD*
P	Inorganic ion transport and metabolism (4/273): *cysI*, *cysP*, *ppK*, *pstC*
Cellular processes	
M	Cell wall/membrane/envelope biogenesis (1/227): *wzc*
O	Posttranslational modification, protein turnover, chaperones (2/144): *clpA*, *cydC*
T	Signal transduction mechanisms (3/186): *csrA*, *csrD*, *yeaP*
U	Intracellular trafficking, secretion, and vesicular transport (1/116): *gspD*
Information, storage and processing	
J	Translation, ribosomal structure and biogenesis (2/188): *pnp*, *prfB*
K	Transcription (6/321): *exuR*, *galS*, *malT*, *mlc*, *spoT*, *yfjR*
L	DNA replication, recombination and repair (3/224):*holC*, *phr*, *recQ*
Poorly characterized	
R	General function prediction only (7/510): *ucpA*, *yafV*, *ybcV*, *yegH*, *yfaY*, *yoaE*, *aspP*
S	Function unknown (4/315): *smg*, *yabI*, *yjcQ*, *yoeB*
	No COG assignment (4/590): *hokA*, *yfdN*, *yhcE*, *ylcG*

Genes are classified into COG categories.[35,36,41] The numbers in parentheses represent the number of glycogen-related genes to the number of genes belonging to each of COG category.

cells of the ASKA library displayed a glycogen-excess phenotype (Fig. 1). It has been shown that RpoS up-regulates the expression of *glgS*, a gene whose product exerts a positive effect on glycogen accumulation.[2,32] Consistently, *glgS* over-expressing cells of the ASKA library displayed a glycogen-excess phenotype (Fig. 1).

3.1.3. 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 mode.[58,59] The hallmark of this pleiotropic physiological response is the accumulation of the alarmones pppGpp and ppGpp.[58–60] Although ppGpp is more abundant than pppGpp, the relative effects of these two regulatory nucleotides have not been thoroughly examined, their levels depending on the synthesis of pppGpp by RelA and SpoT, the hydrolysis of pppGpp to ppGpp by Gpp, and the breakdown of ppGpp by the bifunctional enzyme SpoT.[58–62] (p)ppGpp binds bacterial RNA polymerase to increase transcription of amino acid biosynthesis genes during amino acid starvation and to down-regulate the transcription of 'stable' RNAs (rRNAs and tRNAs) genes.[58,59] As transcription of genes coding for components of the translation apparatus account for a large percentage of transcription in exponentially growing cells, the liberation of RNA polymerase from these genes is thought to passively allow up-regulation of diverse promoters activated at the onset of stationary phase.[63]

Different *in vivo* and *in vitro* experimental evidences have linked the *E. coli* stringent response and (p)ppGpp accumulation with increased glycogen contents and enhanced expression of *glg* genes at the onset of the stationary phase.[25–28] Consistent with the involvement of (p)ppGpp in regulatory aspects of glycogen metabolism, and also consistent with the assigned functions of SpoT and Gpp in (p)ppGpp degradation,[64,65] both *spoT* and *gpp* over-expressing cells of the ASKA collection displayed glycogen-deficient phenotypes (Fig. 1).

We recently found that Δ*relA* cells of the *E. coli* Keio collection[33] display reduced glycogen contents and restricted expression of *glgC*::*lacZ* transcriptional fusions[3] (see also Fig. 2), which further fortifies the view that (p)ppGpp plays an important role in glycogen accumulation in *E. coli*. AG1 strain used in the ASKA library as plasmids recipient has been annotated as a K-12 derivative *relA1* mutant.[34] *relA1* mutants possess little or residual pppGpp synthase activity, which is due to an IS2 insertion between the 85th and 86th codons of the WT *relA* structural gene.[66] It is thus conceivable that *relA* over-expressing cells of the ASKA collection would display a glycogen-excess phenotype. Surprisingly, however, these cells displayed glycogen levels similar to those of control AG1 cells (Fig. 2A and B). To understand why the *relA* over-expressing cells of the ASKA library accumulate glycogen levels comparable to those of control cells, we sequenced the *relA* gene of AG1 cells. This analysis revealed that *relA* of AG1 does not contain any mutation (not shown). To explore whether this phenomenon could be ascribed to the possible occurrence of secondary mutations in AG1 cells or to defects of the ASKA library *relA* expression vector, we analyzed the glycogen contents in Δ*relA* cells of the Keio mutant collection transformed with the ASKA library *relA* expression vector. These cells were constructed on a K-12 derivative BW25113 strain, which is normal for the *relA* function. As shown in Fig. 2C and D, ectopic expression of *relA* complemented the glycogen-deficient phenotype of Δ*relA* cells, the overall data thus showing that (i) AG1 cells are not *relA1* mutants, (ii) the *relA* expression vector of

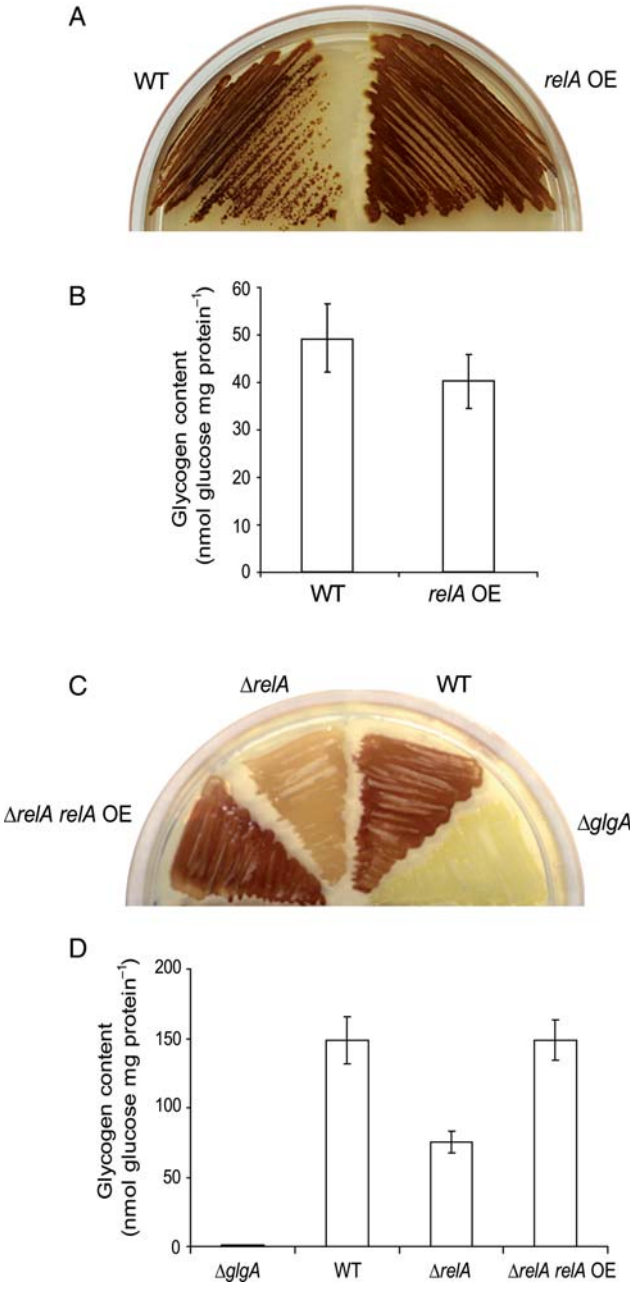

Figure 2. AG1 is not a *relA1* mutant. In (A) and (B), *relA* over-expressing cells of the ASKA library accumulate WT glycogen content. In (C) and (D), *relA* expression vector of the ASKA library complements the glycogen-deficient phenotype of Δ*relA* cells of the Keio collection. Δ*glgA* cells of the Keio collection are used as negative control for glycogen accumulation.

the ASKA library codes for an active RelA form and (iii) ectopic expression of *relA* does not lead to enhancement of glycogen accumulation.

3.1.4. Factors determining intercellular communication, aggregative and social behaviour modes We have recently shown that glycogen metabolism may also be subjected to regulation by cell-to-cell communication.[20] In *E. coli*, swimming, swarming and adherence of cells to surfaces or to one another by biofilm formation are fundamental modes to communicate and to coordinately regulate metabolic processes. Communication, aggregative and social behaviour modes are highly determined by environmental cues and act as major determinants of the nutritional status of the cell, which as discussed above is a major determinant of glycogen accumulation. The following data provided evidence that factors determining intercellular communication, aggregative and social behaviour (Supplementary Fig. S1) are important determinants of glycogen content in *E. coli*, although further studies are required to get a clear picture of the link between the different factors involved.

- First, enhanced expression of the poorly characterized *yncC*, *yncG* and *ymgC* genes (all down-regulated in the *mqsR* biofilm deficient mutants[67]) resulted in increased glycogen content (Fig. 1). *yncC* encodes a transcription factor that positively affects biofilm formation by repressing production of the biofilm matrix component colanic acid,[68] whereas *ymgC* is an orphan gene belonging to the *ymgABC* operon whose transcription is repressed in young and mature biofilms, but is induced in the intermediate, developed biofilms.[69] Although the function of *ymgC* is still unknown, *ymgA* and *ymgB* strongly promote the synthesis of colanic acid, and virtually eliminate the expression of adhesive curli fimbriae genes.
- Second, gaining-of-function of GGDEF and EAL domain enzymes controlling the intracellular levels of cyclic diguanylate (a secondary messenger that regulates the transition from the motile, planktonic state to sessile, community-based behaviours in different bacteria[39,70,71]) resulted in changes in the intracellular glycogen content. For instance, up-regulation of YeaP (a diguanylate cyclase that positively regulates the expression of *csg* genes involved in curli and cellulose production[72]) exerted a negative effect on glycogen accumulation (Fig. 1). In contrast, up-regulation of YjcC (a predicted cyclic diguanylate phosphodiesterase that down-regulates the expression of the CsgD central regulator of extracellular matrix components[37,38,73]) and Dos (a cyclic diguanylate phosphodiesterase[74]) resulted in enhanced glycogen content (Fig. 1).
- Third, some ASKA clones ectopically expressing functions that participate in the synthesis of biofilm components and/or precursors displayed glycogen deficient phenotypes. Thus, ectopic expression of Wzc (an autophosphorylating protein-tyrosine kinase that prevents the production of colanic acid[75,76]), GalS (a repressor of metabolism of galactose linked to the synthesis of

colanic acid and other exopolysaccharide components[77,78]), and NagB (a D-glucosamine 6-*P* isomerase that prevents the synthesis of major components of the cell envelope such as peptidoglycans and lipopolysaccharides[79]) resulted in a glycogen-deficient phenotype (Fig. 1).

3.1.5. Nitrogen metabolism It is known that carbon metabolism is subject to regulation by nitrogen availability, although the mechanisms involved are still obscure. PtsN is a member of the nitrogen-related PTS, which has been associated with balancing of nitrogen and carbon metabolism,[80] and regulation of the RpoE-dependent cell envelope stress response and potassium uptake.[81,82] Most recently, evidence has been provided suggesting the occurrence of cross-talk between sugar-PTS and nitrogen-related PTS.[83] Consistent with this view, the *ptsN* overproducing cells of the ASKA library displayed a marked glycogen-deficient phenotype when cultured in glucose Kornberg medium (Fig. 1).

Yeast extract (the amino acid source of the Kornberg medium employed in this work) is deficient in various amino acids.[2,84] Mutants impaired in cysteine biosynthesis entering the stationary phase display a glycogen-excess phenotype when they are cultured in the Kornberg medium, which is ascribed to stringent response-mediated up-regulation of *glg* genes occurring when the culture medium is deficient in cysteine precursors.[2] Consistent with the view that the stringent response favourably affects glycogen accumulation in *E. coli*, *serB*, *cysI* and *cysP* over-expressing cells of the ASKA library (all ectopically expressing genes involved in cysteine biosynthesis) accumulated lower levels of glycogen than WT cells at the onset of the stationary phase (Fig. 1). *tdcA* positively regulates the expression of *tdcB* and *tdcC* genes, which code for biodegradative proteins involved in threonine and serine metabolism.[85,86] Also consistent with the view that the lack of internal amino acid provision positively affects glycogen accumulation, *tdcA* over-expressing cells of the ASKA library displayed a glycogen-excess phenotype (Fig. 1).

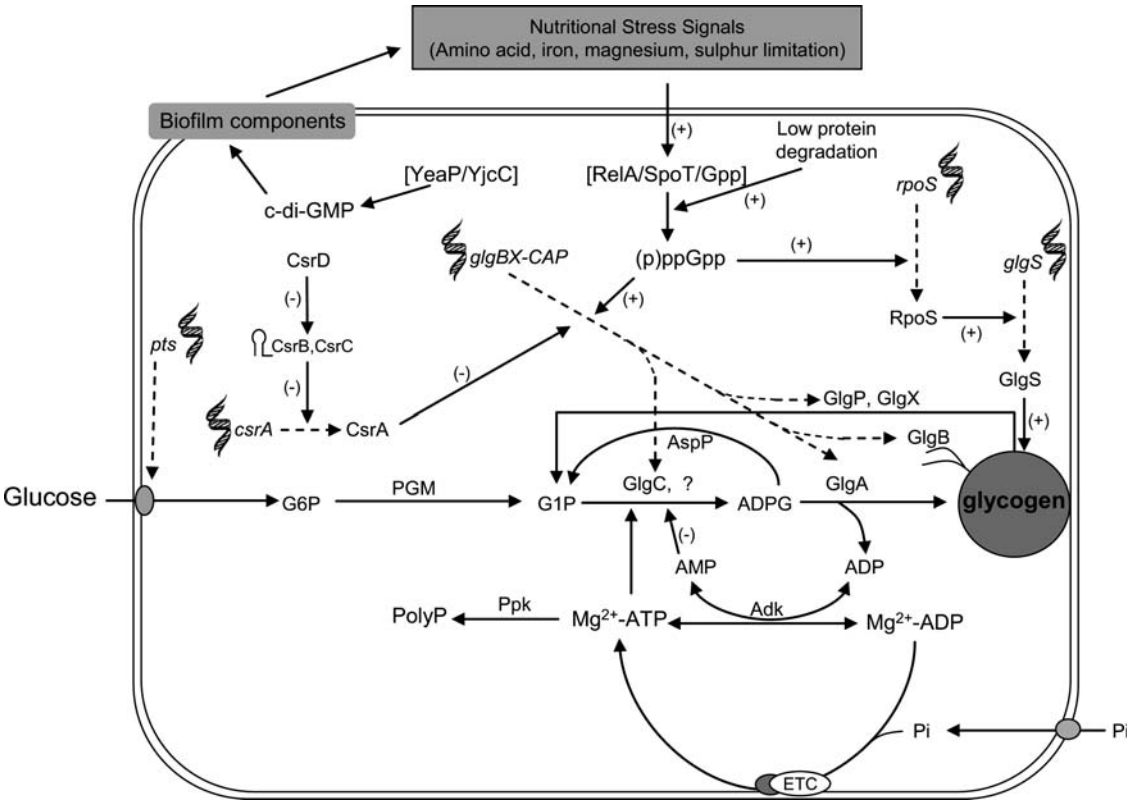

Figure 3. Suggested model of glycogen metabolism in *E. coli* wherein major determinants of glycogen accumulation include availability of ATP for ADPG synthesis, levels of (p)ppGpp (which accumulates in a RelA- SpoT- and/or Gpp-dependent manner under conditions of limited provision of nutrients such as amino acids, sulphur, Mg^{2+}, iron, etc.), factors determining intercellular communication, aggregative and social behaviour modes (which in turn determine the nutritional status of the cell), expression levels of the general stress regulator RpoS and of the global regulator CsrA, 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. CsrB and CsrC small non-coding RNAs, represented by stem-loops, are likely involved in the regulation of functions strongly affecting glycogen accumulation through interaction with CsrA (Adk, adenylate kinase; c-di-GMP, cyclic diguanylate; ETC, electron transport chain).

3.1.6. Energy status Because intracellular ATP level is a major determinant of glycogen accumulation,[3,18] it is conceivable that any factor affecting ATP availability will also affect glycogen accumulation. In fact, deletion mutants lacking components required for the proper functioning of the aerobic electron transport chain and ATP generation displayed a glycogen-deficient phenotype.[2,3] Consistent with this view, bacteria with enhanced expression of cytosolic enzymes likely competing with GlgC for the same ATP pool, such as RecQ (an ATP-dependent DNA helicase[87]), NagD (a promiscuous ribo and deoxyribonucleoside tri-, di- and monophosphatase[88]), and Ppk (an ATP requiring enzyme that catalyzes the production of polyphosphate[89]) displayed glycogen-deficient phenotypes (Fig. 1).

Glutathione is a major determinant of cell redox status, playing a prime role in maintaining the correct assembly of electron transport chain components.[90] It is therefore conceivable that factors altering the intracellular glutathione levels will also affect ATP and glycogen formation. In agreement with this presumption, bacteria with enhanced expression of GorA (a glutathione reductase) and CydC (a protein involved in the transport of glutathione from the cytosol to the periplasm[90]) displayed glycogen deficient phenotypes (Fig. 1).

3.2. *Proposal of an integrated model for the regulation of glycogen metabolism in E. coli*

Results presented in this work further strengthen the view that glycogen metabolism is highly interconnected with a wide variety of cellular processes.[2,3] Figure 3 illustrates a suggested model of glycogen metabolism in *E. coli* wherein major determinants of glycogen accumulation include intracellular concentration and availability of ATP for ADPG synthesis, levels of ppGpp (which accumulates in a RelA- SpoT- and/or Gpp-dependent manner under conditions of limited provision of nutrients such as amino acids, sulphur, Mg^{2+}, iron, etc.), factors determining intercellular communication, aggregative and social behaviour modes (which in turn determine the nutritional status of the cell), expression levels of the general stress regulator RpoS and of the global regulator CsrA, availability of a carbon source and less well-defined systems sensing the cell energy status through the activity of the electron transport chain.

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