REVIEW ARTICLE

Regulation of glycogen metabolism in yeast and bacteria

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Abstract

Microorganisms have the capacity to utilize a variety of nutrients and adapt to continuously changing environmental conditions. Many microorganisms, including yeast and bacteria, accumulate carbon and energy reserves to cope with the starvation conditions temporarily present in the environment. Glycogen biosynthesis is a main strategy for such metabolic storage, and a variety of sensing and signaling mechanisms have evolved in evolutionarily distant species to ensure the production of this homopolysaccharide. At the most fundamental level, the processes of glycogen synthesis and degradation in yeast and bacteria share certain broad similarities. However, the regulation of these processes is sometimes quite distinct, indicating that they have evolved separately to respond optimally to the habitat conditions of each species. This review aims to highlight the mechanisms, both at the transcriptional and at the post-transcriptional level, that regulate glycogen metabolism in yeast and bacteria, focusing on selected areas where the greatest increase in knowledge has occurred during the last few years. In the yeast system, we focus particularly on the various signaling pathways that control the activity of the enzymes of glycogen storage. We also discuss our recent understanding of the important role played by the vacuole in glycogen metabolism. In the case of bacterial glycogen, special emphasis is placed on aspects related to the genetic regulation of glycogen metabolism and its connection with other biological processes.

Introduction

Many microorganisms accumulate carbon and energy reserves to cope with the starvation conditions temporarily present in the environment. The biosynthesis of glycogen is a main strategy for such metabolic storage. 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. In bacteria, the average length of the chains is 8–12 glucose units, and the molecular size of glycogen has been estimated to be about 10^7–10^8 Da. The structure of yeast glycogen is similar to that of other glycogens, with a chain length of 11–12 glucose residues (Northcote, 1953) and a particle diameter of around 20 nm (Mundkur, 1960). In yeast, glycogen is formed upon limitation of carbon, nitrogen, phosphorous or sulfur (Lillie & Pringle, 1980), whereas in bacteria, this polyglucan accumulates under conditions of limiting growth when an excess of carbon source is available and other nutrients are deficient (Lillie & Pringle, 1980; Eydallin *et al.*, 2007b; Montero *et al.*, 2009). An exception to this, where glycogen accumulates to optimal levels during the exponential growth phase, has been observed with cultures of *Streptococcus mitis* (Gibbons & Kapsimalis, 1963) and *Rhodopseudomonas capsulata* (Eidels & Preiss, 1970). In yeast, significant quantities of glycogen are synthesized and degraded as diploid yeast cells undergo the sporulation process (Colonna & Magee, 1978).

One outstanding advantage in using glycogen as a reserve compound is that this macromolecule has little effect on the internal osmotic pressure of the cell. In yeast, the importance of glycogen reserves in survival during long-term

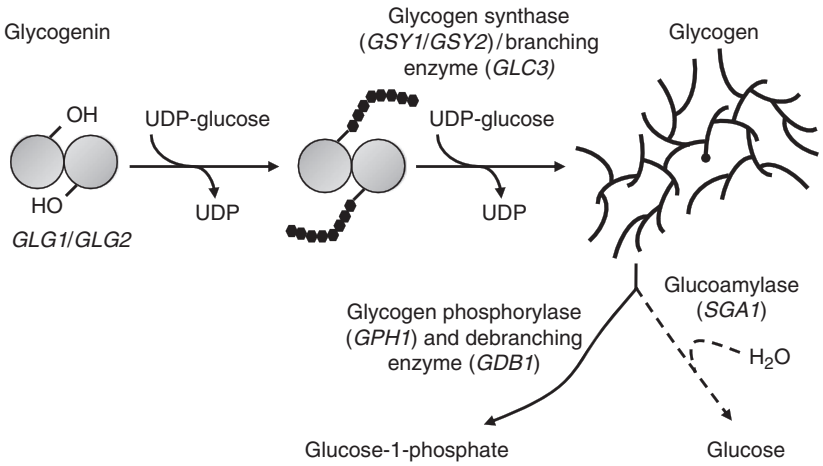

Fig. 1. Schematic representation of the pathways of glycogen synthesis and degradation in yeast. The initiator protein, glycogenin, attaches a glucose residue from UDPG to a tyrosine residue within its own sequence. Glycogenin then adds additional glucose residues, in α-1,4-glycosidic linkage, forming a short oligosaccharide. This oligosaccharide serves as a primer for glycogen synthase, which catalyzes bulk glycogen synthesis by processively adding additional glucose residues in α-1,4-glycosidic linkage. The branching enzyme introduces the α-1,6-branch points characteristic of glycogen. Degradation occurs via the concerted action of glycogen phosphorylase, which releases glucose as glucose-1-phosphate from linear α-1,4-linked glucose chains, and the debranching enzyme, which eliminates the α-1,6-branch points. Alternatively, glycogen can be hydrolyzed in the vacuole by a glucoamylase activity, generating free glucose. See text for details.

nutrient deprivation has been demonstrated clearly (Sillje *et al.*, 1999). Furthermore, yeast cells that can accumulate glycogen stores have a growth advantage over cells that cannot, suggesting that glycogen makes a contribution toward overall fitness (Anderson & Tatchell, 2001). The exact role of this polyglucan in bacteria is not as clear-cut as in animal and yeast cells, but several studies have linked bacterial glycogen metabolism to environmental survival, symbiotic performance and colonization and virulence (Bonafonte *et al.*, 2000; Marroquí *et al.*, 2001; Henrissat *et al.*, 2002; Lepek *et al.*, 2002; Chang *et al.*, 2004; McMeechan *et al.*, 2005; Jones *et al.*, 2008; Sambou *et al.*, 2008; Bourassa & Camilli, 2009). Experiments carried out early in the 1960s suggested that bacteria need glycogen to provide energy for maintenance under nongrowing conditions.

Several excellent reviews on yeast storage carbohydrate metabolism have been published. The most recent dates from 2001 (Francois & Parrou, 2001) and, in the 8 or so years since its publication, some significant advances in our understanding of glycogen storage have been made. For a comprehensive review of the older literature, the reader is referred to Francois & Parrou (2001). Ballicora *et al.* (2003) and Preiss (2009) have published reviews on aspects relating to the allosteric properties and structure–function relationships of enzymes directly involved in bacterial glycogen metabolism, to which readers are referred for further information. Our aim here is to focus on selected areas where the greatest increase in knowledge has occurred.

The regulation of glycogen metabolism in yeast

The enzymes of yeast glycogen storage and utilization

The synthesis of glycogen requires the activities of glycogenin, a self-glucosylating initiator protein (Farkas *et al.*, 1991; Cheng *et al.*, 1995), glycogen synthase (Farkas *et al.*, 1991), which catalyzes bulk synthesis, and the branching enzyme, which introduces the branches characteristic of the mature polysaccharide (Rowen *et al.*, 1992) (Fig. 1). Both glycogenin and glycogen synthase utilize UDP-glucose (UDPG) as a glucose donor and the first step of glycogen synthesis is therefore the generation of UDPG.

UDPG pyrophosphorylase

UDPG is synthesized from UTP and glucose-1-phosphate. This reaction is catalyzed by the enzyme UDPG pyrophosphorylase (EC 2.7.7.9), which is encoded by a single gene, *UGP1* (Daran *et al.*, 1995). The *UGP1* gene is essential due to the pivotal role played by UDPG in yeast metabolism. Protein *N*-glycosylation, utilization of galactose as a carbon source, production of trehalose and synthesis of cell wall β-glucan all require UDPG (Daran *et al.*, 1997) (Fig. 2).

During the logarithmic growth of cells, there is a large demand for UDPG for cell wall β-glucan synthesis to be

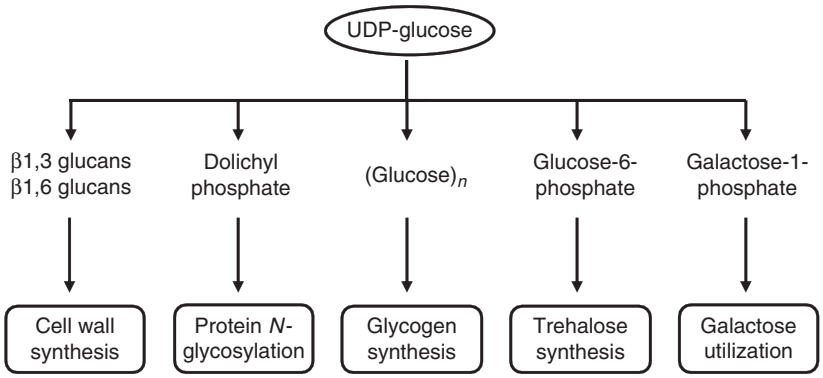

Fig. 2. The various fates of UDPG in yeast metabolism.

maintained. This substantial UDPG demand arises because each new daughter cell clearly requires its own cell wall. However, as cells enter the stationary phase of growth and cell division slows, glycogen synthesis is initiated. What mechanisms regulate the channeling of UDPG between these various processes? Recent work from Rutter's group has demonstrated convincingly the existence of signaling pathways that coordinate channeling of UDPG into either glycogen synthesis or the production of cell wall material in response to both nutritional status and cell integrity signaling (Grose *et al.*, 2007; Smith & Rutter, 2007).

Rutter *et al.* (2002) identified two yeast kinases, Psk1p and Psk2p, as important for growth in a galactose-containing medium at elevated temperature. These kinases contain a conventional serine/threonine kinase domain associated with two PAS domains, and are part of a family of enzymes conserved from yeast to humans (Rutter *et al.*, 2001, 2002; Amezcua *et al.*, 2002). The PAS domains are conserved signaling modules that act as sensors for a wide variety of stimuli, including oxygen, light and small-molecule ligands (Taylor & Zhulin, 1999). Recombinant Psk2p was shown to phosphorylate Ugp1p. This phosphorylation did not alter the activity of Ugp1p. However, failure to phosphorylate Ugp1p *in vivo* led to inadequate cell wall β-glucan synthesis, a weakened cell wall and increased glycogen accumulation (Smith & Rutter, 2007). Therefore, phosphorylation altered the fate of the UDPG synthesized by the enzyme. This channeling of UDPG toward specific fates appeared to be achieved through the control of Ugp1p localization. Phosphorylated Ugp1p localized to the plasma membrane, whereas the dephosphorylated enzyme was found in the cytoplasm (Smith & Rutter, 2007). The synthesis of cell wall β-glucans occurs at the plasma membrane, while glycogen synthesis is a cytoplasmic process (Cid *et al.*, 1995; Montijn *et al.*, 1999; Huh *et al.*, 2003; W.A. Wilson, unpublished data). Thus, the phosphorylated and dephosphorylated forms of Ugp1p govern the fate of UDPG by virtue of their localization to the site of β-glucan synthesis or glycogen synthesis, respectively.

Rutter's group has established that the yeast PAS kinases are regulated in response to both cell integrity stress and nutrient conditions (Grose *et al.*, 2007). The regulation is quite complex and, for example, it appears that cell wall stress activates both Psk1p and Psk2p, whereas Psk1p alone responds to nonfermentable carbon sources (Grose *et al.*, 2007).

Glycogenin

Eukaryotic glycogen synthases cannot initiate the synthesis of a glycogen particle *de novo*. Rather, they function to elongate a pre-existing oligosaccharide primer, which is attached to a protein referred to as glycogenin (EC 2.4.1.186). There are two isoforms of glycogenin in yeast, encoded by *GLG1* and *GLG2* (Cheng *et al.*, 1995). The protein products are 67 and 43 kDa, respectively. Despite this significant size difference, the two proteins appear to be functionally redundant and the deletion of either *GLG1* or *GLG2* has no effect on glycogen storage, whereas a *glg1 glg2* double mutant is glycogen-deficient (Cheng *et al.*, 1995).

Although the initiation steps in glycogen storage might appear to be attractive candidates for regulation, there is no good evidence for the post-translational control of glycogenin activity in yeast. The glycogenins are, however, both regulated at the level of transcription, induction of expression being observed at the diauxic shift (Cheng *et al.*, 1995; DeRisi *et al.*, 1997). Glycogenin serves as an initiator of glycogen synthesis, catalyzing two distinct reactions, which are referred to as the self-glucosylation and elongation reactions, respectively (Mu *et al.*, 1996; Roach & Skurat, 1997). In the self-glucosylation reaction, glucose is transferred from UDPG to a particular tyrosine residue within glycogenin, forming an unusual glucose 1-*O*-tyrosyl linkage (Mu *et al.*, 1996; Roach & Skurat, 1997). In the elongation reaction, additional glucose moieties are added in α-1,4-glycosidic linkage, forming a chain of approximately 8–10 glucose residues. Again, the glucose donor is UDPG. The oligosaccharide bound to glycogenin then serves as the

substrate for glycogen synthase (Roach & Skurat, 1997). The crystal structure of rabbit muscle glycogenin was resolved in 2002 (Gibbons *et al.*, 2002). However, precisely how glycogenin catalyzes the distinct reactions of self-glucosylation (formation of a glucose–tyrosine linkage) and elongation (formation of α-1,4-glycosidic linkages) remains unclear.

Glycogen accumulation can be readily assessed in yeast using the simple procedure of exposing colonies of cells growing on the surface of an agar plate to iodine vapor (Chester, 1968). Yeast cells stain brown in proportion to the amount of glycogen that they contain. A wild-type (WT) yeast strain stains brown while a *glg1 glg2* double mutant yeast strain stains yellow, indicating that glycogen is not being produced (see, e.g. Cheng *et al.*, 1995; Hurley *et al.*, 2005; Torija *et al.*, 2005). However, iodine staining of a population of *glg1 glg2* mutant cells revealed that ~2–3% accumulated glycogen (Torija *et al.*, 2005). This glycogen-storage phenotype was reversible and dependent on glycogen synthase. Furthermore, it was considerably enhanced by expression of an activated form of glycogen synthase or by deletion of the *TPS1* gene that encodes trehalose-6-phosphate synthase (Guillou *et al.*, 2004; Torija *et al.*, 2005). The ability to store glycogen was not stably transmitted between mother and daughter cells. The synthesis of glycogen in the absence of glycogenin therefore appeared to be stochastic in nature. Presumably, there must be some gratuitous primer molecule containing an oligosaccharide (a glycoprotein perhaps), to which glycogen synthase added glucose units. This would be a rare event, possibly enhanced by the expression of an activated glycogen synthase.

Glycogen synthase

Just as there are two isoforms of glycogenin, yeast cells also contain two isoforms of glycogen synthase (EC 2.4.1.11), encoded by the *GSY1* and *GSY2* genes, respectively (Farkas *et al.*, 1991). The protein products are 80% identical. The deletion of the *GSY2* gene results in an approximately 90% decrease in glycogen synthase activity during late logarithmic growth (Farkas *et al.*, 1991). The glycogen content of *gsy2* mutant cells is also much reduced relative to WT (Farkas *et al.*, 1991). In view of these observations, Gsy2p has been considered the major form of glycogen synthase and most characterization efforts have focused on this, rather than the Gsy1p isoform.

Transcription of *GSY2* is strongly induced toward the end of the logarithmic growth phase and this induction requires the cyclic AMP (cAMP)-dependent protein kinase pathway (PKA; discussed in more detail below) (Farkas *et al.*, 1991; Hardy *et al.*, 1994). Post-translational control is also extremely important in the regulation of Gsy2p and the enzyme can be inhibited by phosphorylation (Huang & Cabib, 1974; Francois & Hers, 1988). The inhibition by phosphorylation is overcome by the presence of the allosteric activator glucose-6-phosphate (glucose-6-P) (Huang & Cabib, 1974; Francois & Hers, 1988; Hardy & Roach, 1993; Pederson *et al.*, 2000). Glucose-6-P has little effect on the activity of the dephosphorylated enzyme. The activity measured in the presence of a saturating amount of glucose-6-P is therefore referred to as the total activity because it represents the contribution of all the glycogen synthase molecules present regardless of the phosphorylation state. The ability of glucose-6-P to restore activity to phosphorylated and inactivated glycogen synthase forms the basis of the −/+glucose-6-P activity ratio that is used as an index of activation state. A high activity ratio indicates that glycogen synthase is predominantly in the active, dephosphorylated form while a low ratio shows that phosphorylated, inactive glycogen synthase is present (Huang & Cabib, 1974; Francois & Hers, 1988; Hardy & Roach, 1993; Pederson *et al.*, 2000). The control of glycogen synthase by phosphorylation and glucose-6-P is discussed in detail below.

Branching enzyme

As mentioned above, glycogen is a branched polymer. The branching is important for glycogen function because both the synthesis and the degradation of the polymer occur from the nonreducing ends of the α-1,4 chains (Roach *et al.*, 2001). Branching increases the number of ends and hence the rate at which glycogen can be both degraded and resynthesized. Additionally, the water solubility of glucose polymers increases with the extent of branching, and less-branched polymers, such as amylose, are less soluble than glycogen (Smith, 2001).

Glycogen synthase catalyzes the synthesis of only α-1,4 linkages and the α-1,6 branch points are introduced by a carbohydrate-remodeling activity known as the branching enzyme (EC 2.4.1.18), which is encoded by *GLC3* (Rowen *et al.*, 1992). Branching enzyme transfers a block of six or seven glucose residues from the end of a linear chain of glucose residues linked by α-1,4 bonds, and attaches this block via an α-1,6 linkage to a more interior glucose residue (Gunja *et al.*, 1960; Manners, 1971). Deletion of *GLC3* has been reported to result in considerably reduced carbohydrate storage (Rowen *et al.*, 1992). As with glycogen synthase, transcription of *GLC3* is induced at the approach to the stationary phase, concomitant with the onset of glycogen storage (Rowen *et al.*, 1992; DeRisi *et al.*, 1997). There is no evidence for post-translational control of Glc3p and a key regulator of glycogen branching appears to be the balance between the activity of glycogen synthase and that of the branching enzyme (Raben *et al.*, 2001; Pederson *et al.*, 2003; Wilson *et al.*, 2004).

Glycogen phosphorylase, glucoamylase and the debranching enzyme

Degradation of glycogen in yeast can proceed via two different pathways. First, glycogen can be degraded by glycogen phosphorylase (EC 2.4.1.1; *GPH1* gene product), which releases glucose in the form of glucose-1-phosphate from the nonreducing ends of α-1,4-linked chains (Hwang *et al.*, 1989). Second, free glucose can be generated from glycogen via hydrolysis, which is catalyzed by a vacuolar glucoamylase (EC 3.2.1.3, encoded by the *SGA1* gene) (Colonna & Magee, 1978; Yamashita & Fukui, 1985; Pugh *et al.*, 1989). The role of Sga1p has been somewhat underappreciated in the control of glycogen utilization. This is likely due to its original description as a sporulation-specific enzyme, responsible for the mobilization of glycogen reserves in germinating yeast spores (Colonna & Magee, 1978; Yamashita & Fukui, 1985; Pugh *et al.*, 1989). It is now understood that Sga1p plays a role in glycogen metabolism late in the growth period, even in haploid cells (Wang *et al.*, 2001a). A discussion of our current understanding of the importance of this protein is presented below.

Expression of *GPH1* is induced as cells approach the stationary phase, similar to many other genes involved in glycogen storage and/or utilization (Hwang *et al.*, 1989). As with glycogen synthase, this increase in expression requires the activity of the PKA pathway (Sunnarborg *et al.*, 2001). Yeast glycogen phosphorylase is activated by phosphorylation (Fosset *et al.*, 1971; Lerch & Fischer, 1975; Francois & Hers, 1988). The metabolite glucose-6-P plays a key role in the control of yeast glycogen phosphorylase, this compound serving to facilitate dephosphorylation and inactivation of the enzyme (Lin *et al.*, 1996). There have been some notable developments in our understanding of the regulation of glycogen phosphorylase in yeast since the field was last reviewed and these newer findings are discussed below.

Yeast glycogen phosphorylase is capable of removing glucose residues only from α-1,4-linked glucose chains. The enzyme is incapable of dealing with α-1,6 branch points and indeed stops two to three glucose residues away from them. A debranching enzyme (EC 2.4.1.25; encoded by *GDB1*) is required to handle the branch points (Teste *et al.*, 2000). Gdb1p catalyzes two sequential reactions. First, the maltotriose (or maltose) unit is transferred from the branch point to the nonreducing end of an adjacent α-1,4-linked glucose chain. Second, the residual α-1,6-linked glucose residue is hydrolyzed. Phosphorylase activity then proceeds, degrading glycogen until the next branch point is reached (Lee *et al.*, 1970; Tabata & Hizukuri, 1992; Teste *et al.*, 2000). As would be predicted, deletion of *GDB1* impairs the ability of yeast to mobilize glycogen (Teste *et al.*, 2000). There is no evidence for post-translational control of branching enzyme activity in yeast. Similar to the other enzymes of glycogen metabolism, the expression of *GDB1* is increased at the approach to the stationary phase in batch culture (Teste *et al.*, 2000). The end product of glycogen catabolism via the concerted action of glycogen phosphorylase and the debranching enzyme is a mixture comprising mostly glucose-1-phosphate and also a small quantity of glucose.

Transcriptional control of glycogen storage and utilization

As indicated above, the expression of glycogen synthase, glycogen phosphorylase, the branching enzyme, the debranching enzyme and glycogenin is tightly regulated. The expression of the genes encoding each of these enzymes is induced as cells approach the stationary phase of growth in batch culture and the PKA pathway is central to this control. Mutations that activate PKA result in decreased glycogen storage, whereas mutations that downregulate the PKA pathway result in hyperaccumulation of glycogen (Tatchell *et al.*, 1985; Toda *et al.*, 1985, 1987; Cannon *et al.*, 1986; Cannon & Tatchell, 1987; Levin *et al.*, 1988; Tanaka *et al.*, 1990). As described above, and discussed in detail by Francois & Parrou (2001), the PKA pathway controls transcription of the *GSY1*, *GSY2*, *GLG1*, *GLG2*, *GPH1*, *GLC3* and *GDB1* genes. In the last few years, there has been a significant improvement in our understanding of the transcriptional control of the *GSY2* and *GSY1* genes and this will be discussed below. Figure 3 provides a summary of our current understanding of the promoter regions of *GSY2* and *GSY1*. The reader is referred to Francois & Parrou (2001) for a discussion of historical findings and a comprehensive listing of references.

Control of GSY2 expression by the PKA pathway

PKA-mediated transcriptional control of *GSY2*, and indeed probably all genes regulated by PKA, involves at least in part the transcription factors Msn2p and Msn4p (reviewed by Francois & Parrou, 2001 in relation to glycogen storage). These are largely redundant proteins that are key regulators of the general stress response in yeast (Martinezpastor *et al.*, 1996). Deletion of either *MSN2* or *MSN4* yields a cell with no obvious phenotypic change from the WT (Estruch & Carlson, 1993). However, deletion of both *MSN2* and *MSN4* yields a cell that is hypersensitive to stress and unable to synthesize glycogen (Estruch & Carlson, 1993). Msn2p and Msn4p exert their function through binding to a short DNA motif, known as the STRE element, present in the promoter of around 200 or so stress-sensitive genes (Estruch, 2000; Causton *et al.*, 2001).

Under basal conditions, Msn2p and Msn4p are maintained in the cytoplasm (Gorner *et al.*, 1998). Stress

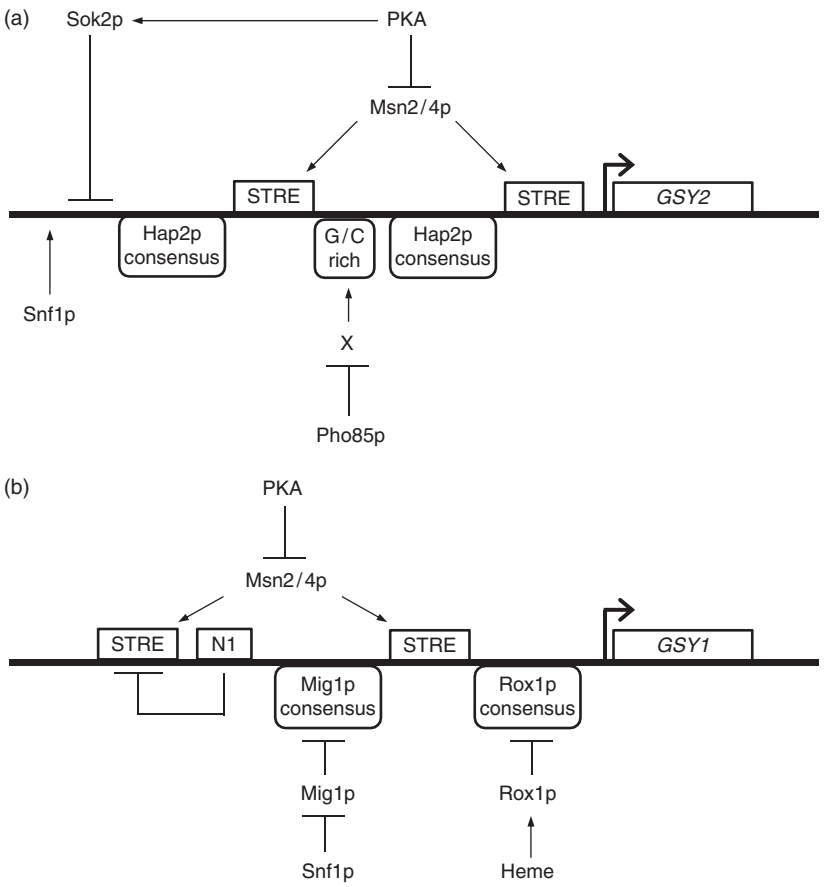

Fig. 3. Models for the transcriptional control of the *GSY1* and *GSY2* genes. (a) The PKA pathway inhibits *GSY2* transcription in part through the Msn2/Msn4p transcriptional activators, which bind to STRE elements in the *GSY2* promoter. There is also Msn2/4p-independent, STRE-independent control by PKA, via the transcriptional repressor Sok2p. The means by which Sok2p represses *GSY2* transcription is undefined. Pho85p represses *GSY2* transcription, likely through inhibition of an unidentified transcriptional activator, X, interacting with the G/C-rich region. The Pcl protein participating in this process is unidentified. Snf1p activates *GSY2* transcription in an indirect fashion via a pathway that requires Mig1p, but not the Mig1p-binding sites in the *GSY2* promoter (which overlap with the G/C-rich region). The *GSY2* promoter contains potential binding sites for Hap2p, but the effect of oxygen on transcription has not been investigated in detail. (b) As with *GSY2*, the PKA pathway inhibits *GSY1* transcription through Msn2/Msn4p and STRE elements in the *GSY1* promoter. Snf1p activates *GSY1* transcription in a process that requires Mig1p- and Mig1p-binding sites in the *GSY1* promoter. A Rox1p-binding site is present and *GSY1* transcription is reduced under aerobic conditions. An additional negatively acting element, N1, is also found, which can repress the function of upstream activating sequences, such as STRE, through an undefined mechanism. Negative regulation by Pho85p has also been reported, but mechanistic details are lacking.

conditions allow translocation into the nucleus and subsequent activation of Msn2p/Msn4p-sensitive genes (Gorner *et al.*, 2002). The nuclear translocation is controlled, at least in part, by the PKA pathway and is best characterized for Msn2p. A current model holds that Msn2p is directly phosphorylated by PKA under basal conditions, maintaining the protein in the cytoplasm (Gorner *et al.*, 2002). The entry of Msn2p into the nucleus takes place subsequent to a reduction in Msn2p phosphorylation, which occurs either through downregulation of the PKA pathway or activation of specific protein phosphatases (Gorner *et al.*, 2002; De Wever *et al.*, 2005).

The promoter of the *GSY2* gene contains two STRE elements (Ni & Laporte, 1995). Francois and colleagues demonstrated that the deletion of these two elements abolished the induction of *GSY2* expression that is normally seen in response to nitrogen starvation, glucose starvation or heat stress (Parrou *et al.*, 1999b). However, removal of the STRE elements did not block the increase in the transcription of *GSY2* that occurred upon the approach to the stationary phase (although the magnitude of the induction was reduced around 20-fold) (Parrou *et al.*, 1999a). Additionally, a yeast strain in which the *GSY2* gene was replaced by a mutant form lacking STRE elements in the promoter was constructed. This yeast strain initiated glycogen synthesis during growth, ultimately accumulating levels similar to WT cells and did so over a similar time course (Enjalbert *et al.*, 2004). The total glycogen synthase activity in the strain with the mutant promoter was less than that seen in WT cells, but, again, the glycogen synthase activity was found to increase during growth, with kinetics indistinguishable from those observed in the WT strain (Enjalbert *et al.*, 2004). In addition, when the STRE elements were removed from the *GSY2* promoter, *GSY2* expression was still responsive to Msn2p/Msn4p (Enjalbert *et al.*, 2004). The control of *GSY2* expression by the PKA pathway was therefore shown to involve at least both STRE-dependent and -independent, and Msn2p/Msn4p-dependent and -independent inputs. One of the STRE-independent, Msn2p-independent pathways appeared to operate through the basic helix–loop–helix transcription factor Sok2p, which was originally identified as a suppressor of the growth defect that arises due to a lack of PKA activity (Ward & Garrett, 1994).

Control of GSY2 expression by the SNF1 pathway

As with many other genes involved in the metabolism of carbohydrates, *GSY2* is known to be subject to glucose repression, that is, transcription is downregulated in the presence of adequate glucose (Hardy *et al.*, 1994). Relief from glucose repression requires the activity of a protein kinase, Snf1p, and the deletion of *SNF1* prevents the derepression of glucose-repressed genes (Hedbacker & Carlson, 2008; Turcotte *et al.*, 2010). A major target of Snf1p is the Mig1p transcriptional repressor (reviewed recently in Turcotte *et al.*, 2010). Phosphorylation of Mig1p by Snf1p results in its inactivation, allowing the derepression of glucose-repressed genes. Transcription of *GSY2* is somewhat reduced in *snf1* mutants, consistent with a role for Snf1p in the regulation of *GSY2* expression (Hardy *et al.*, 1994). The *GSY2* promoter contains putative consensus Mig1p-binding sites and deletion of the *MIG1* gene in an *snf1* mutant yeast strain restored the expression of *GSY2* to WT levels (Enjalbert *et al.*, 2004). However, this effect is unlikely to be direct because oblation of the potential Mig1p-binding sites from the *GSY2* promoter is reported not to impact Snf1p-mediated regulation of expression (Enjalbert *et al.*, 2004). Cells in which the *SNF1* gene is deleted have considerably reduced levels of glycogen relative to WT cells. This reduction in glycogen is more severe than can be accounted for by the modest reduction in *GSY2* transcription observed. Indeed, as discussed below, Snf1p has multiple inputs into glycogen storage beyond its role in the transcriptional control of *GSY2*.

Control of GSY2 expression via the cyclin-dependent kinase Pho85p

The *PHO85* gene encodes the catalytic subunit of a cyclin-dependent protein kinase (Toh-e *et al.*, 1988). Cells in which the *PHO85* gene is deleted grow slowly, show constitutive expression of secreted acid phosphatases, cannot sporulate when diploid, show a defect in growth on nonfermentable carbon sources and have an aberrant morphology including a very large vacuole (reviewed in Huang *et al.*, 2007). In addition, *pho85* mutants also overaccumulate glycogen (Huang *et al.*, 1996b; Timblin *et al.*, 1996). (*Strain-to-strain variability and Pho85p*: It is worth pointing out here that the deletion of *PHO85* has different effects on glycogen accumulation in different genetic backgrounds. In particular, Goding and colleagues have shown that deletion of *PHO85* in a W303a background does not result in hyperaccumulation of glycogen. The majority of studies discussed in this review article use the EG328-1A background, which is a derivative of S288c. Here, the deletion of *PHO85* results in robust glycogen hyperaccumulation.) Pho85p has multiple inputs into glycogen storage, not all of which are conceptually coherent at first sight. The transcriptional inputs are discussed below and additional, post-translational inputs are covered in a later section. Consistent with the pleiotropic role of the Pho85p kinase catalytic subunit, 10 Pho85p cyclins (Pcls) have been identified in yeast (Measday *et al.*, 1997). The hypothesis is that the Pcls confer specificity to the catalytic subunit Pho85p (Andrews & Measday, 1998; Huang *et al.*, 1998). The best-characterized Pcl is arguably Pho80p, which targets Pho85p to the transcription factor Pho4p, thereby regulating gene expression in response to phosphate availability (Kaffman *et al.*, 1994, 1998a, b; O'Neill *et al.*, 1996; Komeili & O'Shea, 1999; Lee *et al.*, 2007). Roles for several other Pcl proteins have also been defined (reviewed by Huang *et al.*, 2007).

Transcription of *GSY2* was found to be increased in *pho85* mutant cells (Timblin & Bergman, 1997). This increase in expression was independent of growth phase and *pho85* mutants showed increased *GSY2* expression relative to WT cells both in the logarithmic phase and at the approach to stationary phase (Enjalbert *et al.*, 2004). Deletion analysis of regions of the *GSY2* promoter revealed that a 14 base pair G/C-rich region was apparently required to confer regulation of expression by Pho85p (Enjalbert *et al.*, 2004). The responsible DNA-binding factor has yet to be identified (Enjalbert *et al.*, 2004). Likewise, the cyclin or cyclins with which Pho85p partners to achieve transcriptional regulation of *GSY2* remains to be defined. As discussed below, when additional inputs of Pho85p into glycogen storage are considered, the related proteins Pcl6p and Pcl7p are likely candidates (Wang *et al.*, 2001b).

Regulation of GSY2 in response to oxygen

Binding sites for the Hap2p protein, a component of the Hap2/3/4/5 complex have been identified in the *GSY2* promoter (Parrou *et al.*, 1999a). Hap2/3/4/5 functions as a heme-activated regulator of transcription (Zitomer & Lowry, 1992; McNabb *et al.*, 1995; Parrou *et al.*, 1999a; Schuller, 2003a; McNabb & Pinto, 2005). Heme synthesis is absolutely dependent upon the presence of oxygen and, therefore, the abundance of heme can serve as an indicator of oxygen status. The Hap2/3/4/5 complex is thus an activator of transcription under aerobic conditions. The presence of the Hap2p-binding sites in the *GSY2* promoter indicated that the gene might be activated in response to oxygen availability. Deletion of a putative Hap2p-binding site did indeed reduce the maximal expression of *GSY2* observed at the diauxic shift, albeit by only around 40% (Parrou *et al.*, 1999a). This might seem modest but, given certain features of the control of *GSY1* expression discussed below, the potential impact of oxygen on the control of *GSY2* expression may be worthy of further consideration.

The roles of TOR in regulation of GSY2 transcription

Treatment of yeast cells with rapamycin evoked many of the same responses as does nutrient starvation, including glycogen accumulation (reviewed in De Virgilio & Loewith, 2006). This gave clear indication that the signaling pathways mediated by the TOR kinases had a role to play in the control of glycogen storage. The TOR kinases are key regulators of cell growth and development in response to nutrient availability. A discussion of the many functions of TOR kinases is not within the scope of this article and the reader is referred to De Virgilio & Loewith (2006) and Rohde *et al.* (2008) for recent, very accessible reviews. At least a portion of the TOR input into regulation of glycogen storage likely involves transcriptional control and there is a TOR component to the retention of the Msn2p/Msn4p proteins within the cytoplasm (Beck & Hall, 1999). When TOR activity is decreased in response to nutrient limitation or rapamycin treatment, uptake of Msn2p/Msn4p into the nucleus would be favored. However, TOR is also a key regulator of the process of autophagy (discussed below) and there is crosstalk between the TOR and Snf1p signaling pathways (Kamada *et al.*, 2000; Rohde *et al.*, 2008). Therefore, dissecting the precise role(s) for TOR kinases in the control of glycogen storage will likely prove difficult.

Regulation of GSY1 transcription

As mentioned above, the major isoform of glycogen synthase is encoded by the *GSY2* gene (Farkas *et al.*, 1991). The role of the minor isoform of the enzyme encoded by *GSY1* has remained somewhat enigmatic. Promoter analysis of *GSY1* by the LaPorte group provided evidence that the control of *GSY1* expression was somewhat different from that of *GSY2*, implying that under certain conditions, *GSY1* might make a more substantial contribution toward glycogen synthase activity (Unnikrishnan *et al.*, 2003). The promoter of the *GSY1* gene has been shown to contain STRE elements (Unnikrishnan *et al.*, 2003). As with *GSY2*, a consensus Mig1p-binding site was also found, which confers regulation by the Snf1p-mediated glucose repression system (Unnikrishnan *et al.*, 2003). Differences between the *GSY1* and the *GSY2* promoters emerged, however, because the *GSY1* promoter also contained a binding site for Rox1p (Unnikrishnan *et al.*, 2003). Rox1p functions as a transcriptional repressor of a variety of genes under aerobic conditions (Keng, 1992). The regulation of *GSY1* expression by Rox1p, and the presence of Hap2p-binding sites in the *GSY2* promoter, raises the possibility at least that the two isoforms of glycogen synthase could be regulated differentially by oxygen availability. Under aerobic conditions, the presence of heme would allow Rox1p production and inhibition of *GSY1* expression. Conversely, the heme produced would allow the activation of Hap2/3/4/5 and enhancement of *GSY2* expression. Under anaerobic conditions, the situation would be reversed. In addition to negative regulation by Rox1p, an additional negative regulatory element, referred to as N1, was identified in the *GSY1* promoter (Unnikrishnan *et al.*, 2003). Deletion of the N1 sequence was shown to enhance *GSY1* promoter activity by as much as fivefold. Finally, there is evidence from a microarray study that Pho85p plays a role in the transcriptional repression of *GSY1* (Carroll *et al.*, 2001).

Control of glycogen synthase by phosphorylation and by glucose-6-P

Glycogen synthase is inhibited by reversible protein phosphorylation. Significant advances in our understanding of this control have emerged since the early work of Cabib and colleagues (Rothman-Denes & Cabib, 1970, 1971) and Francois & Hers (1988). *In vivo* labeling and limited proteolysis localized the phosphorylation sites of Gsy2p to the C-terminus of the protein (Hardy & Roach, 1993). The particular sites phosphorylated, Ser650, Ser654 and Thr667, were then identified using a site-directed mutagenesis approach (Hardy & Roach, 1993). Subsequent work has confirmed that all three sites can be phosphorylated *in vivo* (Gruhler *et al.*, 2005).

Pho85p and the cyclins Pcl8p and Pcl10p phosphorylate and inactivate glycogen synthase

A combination of classical biochemistry, genetic screening in yeast and molecular biology revealed that the cyclin-dependent protein kinase, Pho85p, was the catalytic subunit of a glycogen synthase kinase (Huang *et al.*, 1996b; Timblin *et al.*, 1996).

As discussed above, Pho85p is involved in a large variety of cellular processes, ranging from cell cycle progression to the control of acid phosphatase expression (Huang *et al.*, 2007). The specificity of Pho85p kinase action arises through the association of the catalytic subunit with a variety of different cyclins (Pcls) that function as targeting subunits (Measday *et al.*, 1997; Huang *et al.*, 2007). The Pcls can be divided into two families based on the degree of similarity between their cyclin boxes. One family comprises Pcl1p, Pcl2p, Pcl5p, Pcl9p and Clg1p, and these Pcls are suggested to be involved in cell cycle controls. The other family, comprising Pcl6p, Pcl7p, Pcl8p, Pcl10p and Pho80p, is thought to be involved in metabolic controls (Measday *et al.*, 1997).

An interaction between Pcl10p and Gsy2p was detected in two-hybrid studies, implying that Pcl10p might play a role in the phosphorylation of Gsy2p (Huang *et al.*, 1998). Deletion of *PCL10* had little impact on glycogen storage (Huang *et al.*, 1998). However, the *PCL10* gene is most

closely related in sequence to *PCL8* and the deletion of both *PCL8* and *PCL10* resulted in an activation of glycogen synthase (increase in the −/+glucose-6-P activity ratio) and an increase in glycogen storage (Huang *et al.*, 1998). Therefore, Pho85p, in complex with either Pcl8p or Pcl10p, likely formed a glycogen synthase kinase. Formal proof of this hypothesis came from studies of tagged proteins expressed in yeast and from *in vitro* reconstitution experiments performed with purified, recombinant proteins (Huang *et al.*, 1998; Wilson *et al.*, 1999).

It has proven somewhat easier to generate stable recombinant Pcl10p than to produce recombinant Pcl8p (W.A. Wilson, unpublished data) and most work has therefore focused on this cyclin. Tagged Pcl10p, immunoprecipitated from a yeast cell lysate and containing associated Pho85p, could phosphorylate purified glycogen synthase (Gsy2p) (Huang *et al.*, 1998). Furthermore, recombinant Pho85p was successfully expressed in and purified from *Escherichia coli* (Wilson *et al.*, 1999). This recombinant protein exhibited no activity toward Gsy2p by itself. However, robust kinase activity that inactivated Gsy2p was generated if recombinant Pcl10p was also present in the incubation. The recombinant Pho85p–Pcl10p kinase complex phosphorylated Gsy2p to a stoichiometry of approximately 1.3 mol phosphate : mol protein, phosphorylation occurring preferentially at Thr667 and, to a lesser extent, at Ser654 (Wilson *et al.*, 1999; Pederson *et al.*, 2000). The Pho85p–Pcl10p kinase was incapable of phosphorylating Ser650 (Wilson *et al.*, 1999).

As mentioned above, two-hybrid studies had hinted at a physical interaction between Pcl10p and Gsy2p. These results were confirmed using purified recombinant proteins, where it was established that Pcl10p bound directly to Gsy2p (Wilson *et al.*, 1999). Further work *in vivo* using tagged proteins demonstrated that overexpression of Pcl10p could drive much of the cell's pool of Pho85p into association with glycogen particles, presumably via this interaction with Gsy2p (Wilson *et al.*, 1999). In effect, then, Pcl10p functioned as a targeting subunit that could direct the Pho85p catalytic subunit to the appropriate substrate within the cell. These observations were in agreement with previous work, showing that while Pho85p–Pcl10p complexes immunoprecipitated from yeast could phosphorylate Gsy2p effectively, they were poor kinases for another known Pho85p substrate, the transcription factor Pho4p (Huang *et al.*, 1998). Conversely, immunoprecipitated Pho85p–Pho80p complexes were poor Gsy2p kinases, but highly effective at phosphorylating Pho4p (Huang *et al.*, 1998).

Other kinases acting upon glycogen synthase

As mentioned previously, the sites Ser650, Ser654 and Thr667 on Gsy2p (and at least the sites corresponding to Ser650 and Ser654 in Gsy1p) are known to be phosphorylated *in vivo* (Gruhler *et al.*, 2005). We have also shown that Pho85p–Pcl10p is capable of phosphorylating only Ser654 and Thr667 and cannot phosphorylate Ser650 (Wilson *et al.*, 1999). What is the kinase that phosphorylates Ser650? In a genome-wide screen of deletion mutants with altered glycogen storage, it was determined that *yak1* mutant cells had elevated glycogen (Wilson *et al.*, 2002a). The *YAK1* gene encodes a protein kinase of the DYRK (dual specificity, tyrosine phosphorylated and regulated kinase) family (Garrett & Broach, 1989; Kassis *et al.*, 2000). The observed increase in glycogen content upon deletion of *YAK1* implied some role for this kinase in the control of glycogen storage.

Yak1p appears to function in the PKA nutrient signaling pathway and has been reported to translocate from the cytoplasm to the nucleus upon nutrient limitation (Garrett & Broach, 1989; Werner-Washburne *et al.*, 1991; Griffioen *et al.*, 2001). The translocation of Yak1p to the nucleus appears to be TOR dependent and is also stimulated by rapamycin treatment (Schmelzle *et al.*, 2004) and Yak1p functions in the signaling pathway that links TOR activity to the synthesis of ribosomal proteins (Martin *et al.*, 2004). Yak1p also phosphorylates Pop2p, an RNase of the DEDD family (Moriya *et al.*, 2001). Intriguingly, DYRK1A and DYRK2, two mammalian DYRK family kinases that are related to Yak1p, are known to phosphorylate and inactivate the muscle isoform of glycogen synthase (Skurat & Dietrich, 2004). Phosphorylation occurs at the site referred to as 3a, which is clearly related in sequence to Ser650 in Gsy2p (Skurat & Dietrich, 2004). Considering the intimate links between Yak1p and nutrient-sensing systems, it might seem naive to assume that Yak1p would regulate glycogen storage through direct phosphorylation of Gsy2p. However, the similarities between Yak1p and the mammalian DYRK kinases did make this an intriguing hypothesis. Despite resulting in significant glycogen overaccumulation, the deletion of *YAK1* did not cause activation of glycogen synthase, as determined by measurement of the −/+glucose-6-P activity ratio (Wilson *et al.*, 2002a; W. Wilson, unpublished data). Therefore, it appears that Yak1p-mediated phosphorylation likely does not directly affect the catalytic activity of Gsy2p, although this must be tested by *in vitro* phosphorylation experiments.

The PAS kinase Psk2p has also been reported to phosphorylate glycogen synthase and *psk2* deletion mutants show increased glycogen storage (Rutter *et al.*, 2002; Wilson *et al.*, 2002a). In this case, the phosphorylation has been shown to occur at Ser654 (Rutter *et al.*, 2002). However, stoichiometric phosphorylation of Gsy2p using recombinant Pask2p did not alter the activity of glycogen synthase (W.A. Wilson & J. Rutter, unpublished data). As described above, control of glycogen storage via PAS kinases

likely occurs indirectly through the regulation of UDPG channeling.

Phosphatases acting upon glycogen synthase

There have been reports that mutations in several yeast protein phosphatase catalytic subunit genes affect glycogen accumulation (Peng *et al.*, 1991; Posas *et al.*, 1991, 1993; Clotet *et al.*, 1995). However, it is now accepted that the type-1 protein phosphatase, encoded by *GLC7*, is the major phosphatase regulating glycogen storage (Feng *et al.*, 1991). Glc7p carries out many different cellular functions through association with specific accessory proteins, referred to as targeting subunits (Hubbard & Cohen, 1993). *GLC7* is an essential gene because there is a requirement for Glc7p function during mitosis (Hisamoto *et al.*, 1995; Mackelvie *et al.*, 1995). Certain alleles of *glc7*, most notably *glc7-1*, render cells glycogen-deficient (Cannon *et al.*, 1994). Glycogen synthase is heavily phosphorylated and inactive in *glc7-1* mutant cells. The mutation present in the *glc7-1* allele prevents the interaction between Glc7p and a particular targeting subunit known as Gac1p. Gac1p is related in sequence to mammalian R_{GL}, which functions to target the type-1 protein phosphatase catalytic subunit to glycogen particles in skeletal muscle (Stralfors *et al.*, 1985; Tang *et al.*, 1991; Francois *et al.*, 1992; Stuart *et al.*, 1994). Similarly, Gac1p directs Glc7p to glycogen particles in yeast (Stuart *et al.*, 1994).

Poorly defined inputs of the Snf1p and PKA pathways into post-translational control of glycogen synthase

Both Snf1p and PKA have clear transcriptional inputs into glycogen storage. However, both proteins also appear to play roles in the post-translational regulation of glycogen synthase activity (Hardy *et al.*, 1994; Francois & Parrou, 2001). As described above, Gsy2p expression is very low in cells where the PKA pathway is constitutively activated. However, the expression of *GSY2* from a heterologous promoter in a *bcy1* strain can bypass the transcriptional control, allowing a high level of Gsy2p production (Hardy *et al.*, 1994). Glycogen accumulation still does not occur, though, because the Gsy2p is highly phosphorylated and inactive (Hardy *et al.*, 1994). The mechanism by which PKA regulates Gsy2p phosphorylation remains unresolved. In terms of both the transcriptional and the post-translational control of glycogen synthase, there is a clear antagonism between the PKA and the Snf1p pathways. Regarding the post-translational regulation, the deletion of *snf1* results in a similar phenotype to activation of the PKA pathway, which is hyperphosphorylation and inactivation of Gsy2p (Hardy *et al.*, 1994). The Snf1p kinase is itself a phosphoprotein and phosphorylation at Thr210 is required for activation (Sutherland *et al.*, 2003; Elbing *et al.*, 2006; Hedbacker & Carlson, 2008). The inactivation of Snf1p involves the removal of phosphate from Thr210 and is catalyzed by the Glc7p protein phosphatase in complex with the Reg1p targeting subunit (McCartney & Schmidt, 2001). The deletion of *REG1* rendered Snf1p constitutively phosphorylated and active (McCartney & Schmidt, 2001). The deletion of *REG1* also resulted in hyperaccumulation of glycogen, which could not be entirely accounted for by the modest increase in *GSY2* expression resulting from the bypass of glucose repression (Huang *et al.*, 1996a).

Additionally, Snf1p functions as part of a heterotrimer that contains Snf4p and one of three related proteins, collectively referred to as β-subunits, which are encoded by the *SIP1*, *SIP2* and *GAL83* genes (reviewed in Hedbacker & Carlson, 2008). Intriguingly, Sip1p, Sip2p and Gal83p all contain a carbohydrate-binding sequence, which has been referred to as the glycogen-binding domain (Wiatrowski *et al.*, 2004; Mangat *et al.*, 2010). The Gal83p subunit has been shown to bind tightly to glycogen and could therefore provide a means of tethering the Snf1p kinase complex to glycogen particles (Wiatrowski *et al.*, 2004). Mutation of residues implicated in glycogen particle binding resulted in increased glycogen storage as well as activation of transcription of a variety of genes, including *GSY1* and *GSY2* (Wiatrowski *et al.*, 2004). Deletion of the entire glycogen-binding domain from Gal83p produced a constitutively active form of the Snf1p kinase (Mangat *et al.*, 2010). The significance of the binding interaction between the Snf1p kinase complex and glycogen particles is unclear and, indeed, point mutations that influence glycogen binding of the Gal83p subunit have been shown to upregulate the transcription of a variety of Snf1p-regulated genes even in a yeast background that does not synthesize glycogen (Wiatrowski *et al.*, 2004).

The interplay between glucose-6-P and phosphorylation in the control of Gsy2p

As mentioned above, post-translational regulation of glycogen synthase involves the interplay of two regulatory mechanisms, namely inhibition by reversible phosphorylation and activation by the allosteric modulator, glucose-6-P. Because the inhibitory effects of phosphorylation can be overcome by sufficient glucose-6-P, the activity state of glycogen synthase at any particular instance is a function of both the phosphorylation state of the enzyme and the glucose-6-P content of the cell. This interplay is well demonstrated by the observation that the glycogen-deficient phenotype of *snf1* mutant cells, which arises in large part from hyperphosphorylation and inactivation of glycogen synthase, can be suppressed by mutations that elevate

glucose-6-P (Hardy *et al.*, 1994; Huang *et al.*, 1997). In addition to allosteric activation of glycogen synthase, glucose-6-P has been shown to both stimulate the dephosphorylation of glycogen synthase and to inhibit its phosphorylation (Francois & Hers, 1988; Huang *et al.*, 1997). Whether these latter effects are mediated by binding to glycogen synthase or through regulation of kinases/phosphatases that act upon glycogen synthase remains unclear.

The availability of bacterially expressed, highly purified glycogen synthase and Pho85p–Pcl10p kinase complexes allowed a rigorous assessment of the properties of Gsy2p and an analysis of the precise effects of phosphorylation and glucose-6-P on the kinetic parameters of the enzyme (Pederson *et al.*, 2000). Recombinant Gsy2p was found to have a −/+glucose-6-P activity ratio of approximately 0.4–0.5. Therefore, the enzyme, in its completely dephosphorylated state (as isolated from *E. coli*), retained some degree of glucose-6-P dependence (Pederson *et al.*, 2000). Indeed, addition of glucose-6-P reduced the K_m for both UDPG and glycogen and also increased the V_{max}.

Phosphorylation by the Pho85p–Pcl10p kinase complex to a stoichiometry of ~1.3 mol phosphate : mol Gsy2p induced a drastic change in the kinetic properties. The phosphorylated enzyme had a very low activity without glucose-6-P. Under these conditions, the K_m for UDPG was considerably increased and, in fact, could not be determined because it was not possible to reach saturation. An increase in the K_m for glycogen was also observed. When glucose-6-P was added, however, the kinetic properties of the phosphorylated enzyme were comparable to those of the non-phosphorylated enzyme in the presence of glucose-6-P. The ability of glucose-6-P to overcome the effects of phosphorylation allowed the development of a three-state model for the control of enzyme activity, which is illustrated in Fig. 4.

The crystal structure of any fungal or mammalian glycogen synthase is still not available and a complete understanding of regulation by phosphorylation and glucose-6-P must await the publication of such data. However, some progress has been made toward a better appreciation of how glucose-6-P and phosphorylation affect the kinetic properties of the enzyme since the publication of the last major review in this area. Reasoning that glucose-6-P would likely interact with glycogen synthase through binding to positively charged residues, systematic mutagenesis of Gsy2p was carried out, mutating clusters of basic residues to alanine. This approach led to the identification of two triple mutants (R579A/R580A/R582A and R586A/R588A/R591A) that had altered sensitivity to glucose-6-P and somewhat different properties (Pederson *et al.*, 2000). Whereas the R586A/R588A/R591A mutant could be phosphorylated and inactivated similar to the WT enzyme by Pho85p–Pcl10p, this inactivation was not reversed by glucose-6-P addition.

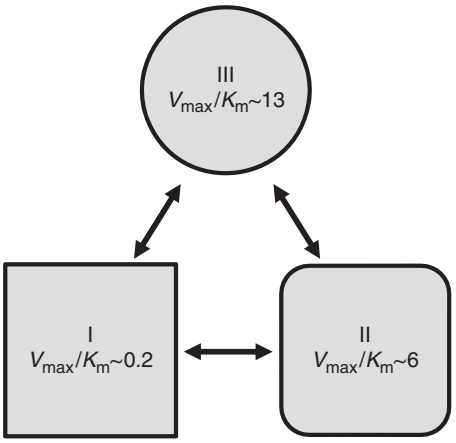

Fig. 4. A three-state model for the regulation of Gsy2p. Gsy2p is proposed to exist in three different activity states. The lowest activity state corresponds to the phosphorylated enzyme in the absence of glucose-6-P (state I). The dephosphorylated enzyme in the absence of glucose-6-P has intermediate activity (state II). Both state I and state II can be converted to the most active state, state III, by binding of glucose-6-P.

The activity of the R579A/R580A/R582A mutant, on the other hand, was reduced relative to the WT enzyme in the absence of phosphorylation. Phosphorylation did still reduce the activity of this mutant, but only slightly. As with the R586A/R588A/R591A mutant, glucose-6-P was unable to restore activity to the phosphorylated enzyme (Pederson *et al.*, 2000). Therefore, the R586A/R588A/R591A mutant was likely deficient in glucose-6-P binding, whereas the R579A/R580A/R582A mutant was probably defective in the transition between different activity states and was unable to reach the highest activity state (Fig. 4). These *in vitro* studies were extended by work in yeast and it was shown that normal glycogen accumulation required the activation of glycogen synthase by glucose-6-P (Pederson *et al.*, 2004).

Regulation of glycogen phosphorylase by phosphorylation

Gph1p is activated by phosphorylation of a specific threonine residue, Thr31, and mutant Gph1p, where this threonine is converted to alanine and is essentially inactive (Lin *et al.*, 1995). The crystal structure of yeast glycogen phosphorylase was resolved in both the phosphorylated and the dephosphorylated forms and an excellent mechanistic understanding of how phosphorylation at Thr31 serves to regulate activity is available (Lin *et al.*, 1996, 1997). *In vitro*, Thr31 can be phosphorylated by PKA, activating the enzyme. However, despite considerable effort and several reports of partial purification, the physiological yeast glycogen phosphorylase kinase has not been identified (Becker *et al.*, 1983; Pohlig *et al.*, 1983). There has been more progress in the identification of Gph1p phosphatases and a

regulatory network that controls the dephosphorylation and inactivation of Gph1p is beginning to be unraveled (Tung *et al.*, 1995; Zhang *et al.*, 1995; Nigavekar *et al.*, 2002; Tan *et al.*, 2003; Wilson *et al.*, 2005). At the center of this network lies Pho85p once again.

Pho85p in the control of the phosphorylation state of glycogen phosphorylase

The connection between glycogen phosphorylase and Pho85p was first established through studies of respiratory mutants and cells lacking functional *SNF1* (Wang *et al.*, 2001b; Wilson *et al.*, 2002b, 2005). Respiratory mutants begin to synthesize glycogen as nutrients in the medium are depleted, just as WT cells do (Chester, 1968; Enjalbert *et al.*, 2000; Wilson *et al.*, 2002b). However, respiratory mutants obviously cannot survive on nonfermentable carbon sources, such as ethanol. When glucose is depleted from the growth medium, such cells are forced to consume their glycogen stores. In contrast, WT cells are able to maintain significant glycogen stores as they transition to oxidative metabolism. Respiratory mutants therefore appear to be glycogen-deficient relative to WT cells (Chester, 1968; Enjalbert *et al.*, 2000; Wilson *et al.*, 2002b). The glycogen storage defect in respiratory mutants is thus due to enhanced glycogen utilization rather than a defect in glycogen synthesis (Enjalbert *et al.*, 2000). As such, one would not expect it to be suppressed by mutation of *PHO85*, which leads to dephosphorylation and activation of glycogen synthase. This was indeed the case and mutation of *PHO85* in respiratory mutants yielded an initial increase in glycogen storage, but could not suppress the utilization of this glycogen as the cells ran out of fermentable sugar (Enjalbert *et al.*, 2000; Wilson *et al.*, 2002b). Indeed, glycogen degradation was actually enhanced in respiratory mutants that also lacked a functional *PHO85* gene. This observation was explained by the finding that the deletion of *PHO85* resulted in a substantial increase in glycogen phosphorylase activity, which was independent of *PCL8* and *PCL10* (Wilson *et al.*, 2002b).

In vitro assays demonstrated that the ability to dephosphorylate and inactivate glycogen phosphorylase was substantially decreased in *pho85* mutant cells (Wilson *et al.*, 2005). Deletion of the related cyclins *PCL6* and *PCL7* resulted in a similar inability to dephosphorylate phosphorylase (Wilson *et al.*, 2005). Therefore, Pho85p–Pcl6p or Pho85p–Pcl7p appeared to be required, either directly or indirectly, to activate a phosphorylase phosphatase. These data were consistent with the role for *PCL6* and *PCL7* in the control of glycogen phosphorylase that had been hinted at in earlier work (Wang *et al.*, 2001b).

To identify the phosphorylase phosphatase, *in vitro* phosphorylase phosphatase assays were performed using yeast deletion mutants, each of which lacked either a protein phosphatase catalytic subunit or a protein phosphatase regulatory/targeting subunit (Wilson *et al.*, 2005). Only strains in which either the *SHP1* or *GLC8* genes had been deleted showed any appreciable reduction in phosphorylase phosphatase activity (Wilson *et al.*, 2005). Shp1p is a member of the ubiquitin regulatory X domain-containing protein family and is known to interact with Cdc48p, a component of the retrotranslocation machinery that moves ubiquitinated proteins from the endoplasmic reticulum into the cytosol (Schuberth *et al.*, 2004). The precise link between Shp1p and Glc7p activity is therefore somewhat obscure, although the deletion of Shp1p had been reported previously to reduce Glc7p activity measured in cell extracts (Zhang *et al.*, 1995).

Glc8p, on the other hand, is well characterized. Glc8p was known to interact physically with Glc7p (Ramaswamy *et al.*, 1998; Ho *et al.*, 2002). Indeed, Glc8p had been shown to function as a Glc7p activator and the deletion of *GLC8* reduced Glc7p activity measured in cell extracts (Tung *et al.*, 1995; Ramaswamy *et al.*, 1998; Nigavekar *et al.*, 2002). Glc8p was known to be a phosphoprotein and phosphorylation was known to be important for Glc8p function *in vivo* (Tung *et al.*, 1995). In an elegant study that combined classical genetic techniques with the use of a library of yeast strains, each expressing one particular protein kinase fused to glutathione-*S*-transferase, Tan *et al.* (2003) clearly established that Pho85p functioned as the physiological Glc8p kinase. Furthermore, these workers also demonstrated that the cyclins responsible for directing Pho85p toward phosphorylation of Glc8p were Pcl6p and Pcl7p, and that phosphorylation of Glc8p by Pho85p–Pcl7p (or Pho85p–Pcl6p) was required for the full activity of the Glc7p phosphatase (Tan *et al.*, 2003).

Taking all of the above observations together, we arrive at a model whereby Pho85p, in conjunction with the cyclins Pcl6p and Pcl7p, functions as a positive regulator of the Glc7p phosphatase. This positive regulation occurs via phosphorylation of Glc8p by a kinase comprising Pho85p and Pcl7p (or Pcl6p). The Glc7p phosphatase then dephosphorylates and inactivates glycogen phosphorylase (Fig. 5).

Spatial aspects of glycogen storage

Glycogen synthesis and degradation have long been considered to be cytoplasmic processes. It is now apparent, however, that separable pools of glycogen exist within the yeast cell and that the vacuole has a key role to play in the long-term maintenance of glycogen stores (Wang *et al.*, 2001a; Wilson *et al.*, 2002a). In addition, the key enzymes of glycogen synthesis and degradation, glycogen synthase and glycogen phosphorylase, show alterations in their subcellular distribution dependent on the glycogen content,

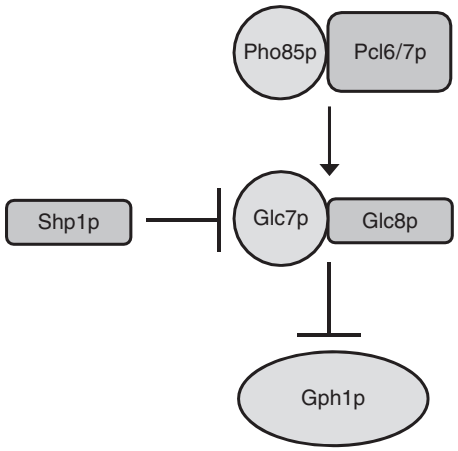

Fig. 5. Control of glycogen phosphorylase activity by Pho85p and Glc7p. Pho85p and the cyclin Pcl6p (or Pcl7p) form a complex that phosphorylates Glc8p. This phosphorylation serves to activate the Glc7p–Glc8p phosphatase complex, which then dephosphorylates and inactivates glycogen phosphorylase. Shp1p appears to be a positive regulator of bulk Glc7p activity, which acts independent of Pho85p, and is not specific to the regulation of phosphorylase phosphatase activity.

glycogen synthase entering the nucleus under certain conditions (W.A. Wilson, unpublished data). Therefore, neither glycogen itself nor the enzymes of its metabolism can be considered purely cytoplasmic. The experiments that led to these conclusions and the significance of the findings to studies of glycogen storage are discussed below.

Vacuolar glycogen stores and autophagy

The first indication that the vacuole might play a role in glycogen metabolism came from the application of a classical genetic screen (Wang *et al.*, 2001a). Although the deletion of the *PHO85* gene restored glycogen accumulation to a strain deleted for the *SNF1* gene, deletion of the two cyclins known to target Pho85p toward glycogen synthase, Pcl8p and Pcl10p, apparently failed to do so and an *snf1 pcl8 pcl10* triple mutant appeared to be glycogen deficient (Huang *et al.*, 1998). A screen for multicopy suppressors of the glycogen storage defect of this triple mutant yielded six genes (Wang *et al.*, 2001a). The gene recovered most frequently was *ATG1*, which encodes a protein kinase required for the process of autophagy (Matsuura *et al.*, 1997).

Similar to glycogen accumulation, autophagy is induced when yeasts are exposed to nutrient-limited conditions. Starvation signals are sensed and passed to a complex of several proteins referred to as the preautophagosomal structure. The preautophagosomal structure then initiates the formation of a double-membrane structure, referred to as the isolation membrane, which expands and envelopes a region of the cytoplasm. Material within this region becomes incorporated into a double-membrane-bound vesicle known as an autophagosome as the leading edges of the isolation membrane fuse together. The autophagosome in turn fuses with the vacuole, releasing a single-membrane-bound autophagic body into the vacuole lumen. The membrane and contents of the autophagic body are then degraded within the vacuole (reviewed in Xie & Klionsky, 2007; Cebollero & Reggiori, 2009). Overexpression of *ATG1* in a WT yeast background led to a modest increase in glycogen (and increased autophagy) (Wang *et al.*, 2001a). Conversely, the deletion of *ATG1* in an otherwise WT background resulted in reduced glycogen storage that became evident only after several days of growth (Wang *et al.*, 2001a). In addition, it was determined that the deletion of *SNF1* inhibited autophagy, whereas the deletion of *PHO85* enhanced the process (Wang *et al.*, 2001a). Therefore, a link between autophagy and glycogen storage capacity had been uncovered, with the ability to carry out autophagy being required for normal glycogen storage.

Careful analysis of the *snf1 pcl8 pcl10* mutant strain revealed that it was capable of glycogen synthesis at the approach to the stationary phase. However, this glycogen was rapidly degraded and thus the strain appeared to be glycogen-deficient later in growth (Wang *et al.*, 2001a). In addition to the effects on glycogen synthase regulation (suppressed by the deletion of both *PCL8* and *PCL10*), the deletion of the *SNF1* gene also inhibited autophagy (Wang *et al.*, 2001a). Enhancement of autophagy by overexpression of *ATG1* somehow restored glycogen storage. Potentially, the ability of *PHO85* deletion to restore essentially normal glycogen accumulation and maintenance of stores to an *snf1* mutant could be partly explained by the enhancement of autophagy observed in this strain as well as the effect on glycogen synthase regulation.

Why would enhancement of autophagy promote retention of glycogen stores? In WT yeast, the synthesis of glycogen begins in the late logarithmic phase and, during the stationary phase, glycogen undergoes partial consumption, between 24 and 48 h, presumably in correspondence with the metabolic reprogramming necessitated by the depletion of glucose and limitation of other nutrients (Francois & Parrou, 2001; Wang *et al.*, 2001a). During this period, glycogen phosphorylase is active and it is at this time that autophagy mutants rapidly deplete their glycogen. In WT strains, there is then a phase of resynthesis and maintenance of glycogen stores up to 5–6 days, whereas autophagy mutants never re-establish high glycogen levels (Wang *et al.*, 2001a). A model has been proposed in which there are two glycogen pools: cytosolic and vacuolar (Fig. 6). Defective autophagy means that no glycogen is delivered to the vacuole and also that no metabolic intermediates are exported from the vacuole. Both could reduce glycogen

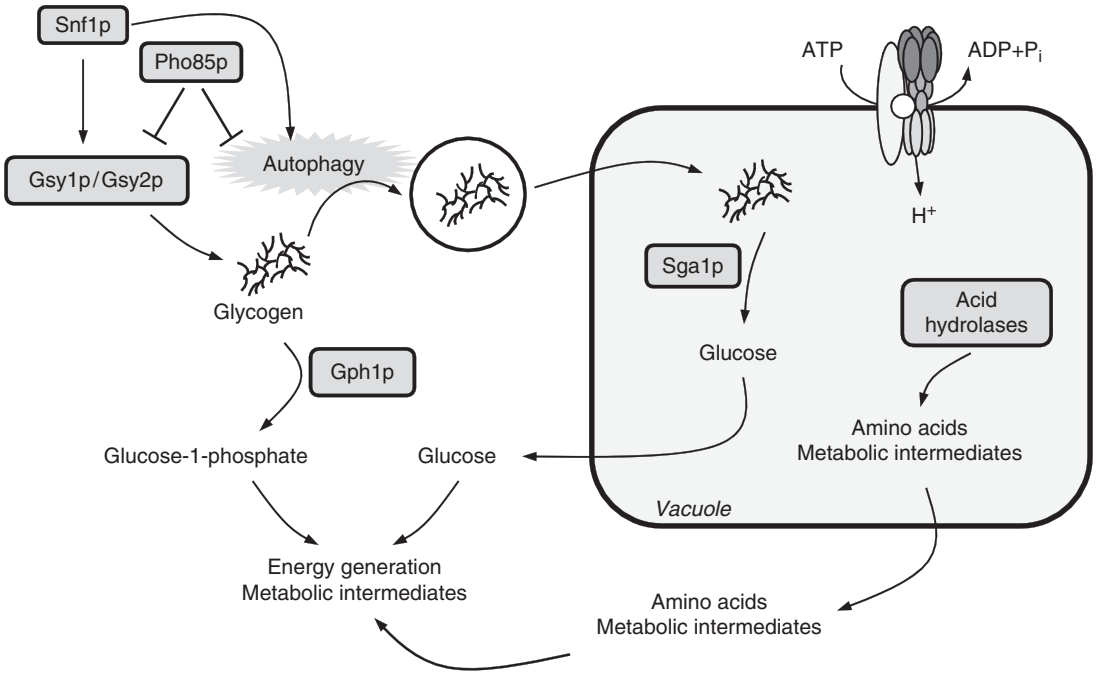

Fig. 6. A model for the relationship between autophagy and glycogen storage. Synthesis of glycogen begins late in the logarithmic phase of growth. The nutrient limitations that trigger glycogen accumulation also enhance autophagy. Snf1p is a positive regulator of glycogen accumulation and also a positive regulator of autophagy. In contrast, Pho85p is a negative regulator of both processes. The contributions of autophagy to glycogen storage are at least twofold. First, the degradation of cellular material provides a source of metabolic intermediates, such as amino acids, which can be passed back into the cytoplasm for reuse. These intermediates may provide a source of energy and/or metabolic intermediates, which could allow preservation (or even resynthesis) of glycogen reserves. Second, autophagy delivers some glycogen to the vacuole, where it is protected from degradation by the cytoplasmic enzyme, glycogen phosphorylase. The vacuolar pool of glycogen can be accessed very late in growth through hydrolysis, catalyzed by Sga1p. In the absence of autophagy, a supply of energy and metabolic intermediates is lost. Furthermore, there is no route by which glycogen can reach the vacuole. The cell's glycogen reserves remain in the cytoplasm and are degraded by glycogen phosphorylase, providing energy and carbon skeletons. In the presence of autophagy, but in the absence of a fully functional vacuole, glycogen reserves are protected. This might reflect an inability of Sga1p to function appropriately in a compromised vacuole.

levels: in the absence of normally recycled intermediates, glycogen may be depleted and there would be no vacuolar glycogen store, protected from cytosolic phosphorylase. This vacuolar glycogen store would normally be accessed later in starvation through the activity of the vacuolar α-glucosidase encoded by *SGA1* (Yamashita & Fukui, 1985; Pugh *et al.*, 1989; Wang *et al.*, 2001a). Despite its description as a sporulation-specific gene, *SGA1* does play a role in the glycogen metabolism of haploid cells during vegetative growth. This was demonstrated by gene deletion studies in which double mutants lacking both the *GPH1* and the *SGA1* genes were constructed. Deletion of *GPH1* resulted in an overaccumulation of glycogen that was most apparent after prolonged growth (Hwang *et al.*, 1989). Deletion of *SGA1*, on the other hand, did not result in an increase in glycogen storage, but did confer inhibition of glycogen degradation very late in the stationary phase (Wang *et al.*, 2001a). Deletion of both *GPH1* and *SGA1* generated a strain that both overaccumulated glycogen in the stationary phase and maintained that glycogen later in growth (Wang *et al.*, 2001a).

Vacuolar acidification and glycogen stores

Further evidence for the role of the vacuole in glycogen storage came from a genome-wide screen for deletion mutants that were either defective in glycogen storage or hyperaccumulated this compound (Wilson *et al.*, 2002a). Of the 566 strains identified that had a glycogen content different from the WT, approximately 10% had functions related to vesicle trafficking or vacuole function. Of particular note, mutants in nine genes encoding structural components of the vacuolar proton-translocating ATPase (V-ATPase) and five genes encoding proteins required for V-ATPase assembly were recovered in the screen. The V-ATPase is a multiprotein complex that is required for the acidification of internal organelles, including the vacuole (reviewed by Kane, 2006). The V-ATPase genes identified

represent an obvious functional cluster, making a robust link between the ability to acidify the vacuole appropriately and glycogen storage. Of the 14 deletion mutants linked to V-ATPase function that were identified as impacting glycogen storage, all except for two resulted in increased glycogen stores. This indicated that a defective V-ATPase resulted in stabilization of the vacuolar glycogen pool. In support of this proposal, a functional pathway of autophagy was found to be required for overaccumulation of glycogen in a *vma10* mutant strain. When autophagy was blocked in the *vma10* mutant strain by deletion of *ATG1*, glycogen overaccumulation was also blocked. Presumably, without its ATPase, the vacuole could not acidify and function normally. In particular, the degradative enzymes of the vacuole would not be fully active. As mentioned above, the glucoamylase encoded by *SGA1* resides in the vacuole and, presumably, its activity would be impaired (Colonna & Magee, 1978; Yamashita & Fukui, 1985; Pugh *et al.*, 1989).

The subcellular localization of glycogen synthase and glycogen phosphorylase

Over the past 15 years or so, evidence from mammalian systems has been steadily accumulating, demonstrating that the subcellular distribution of glycogen synthase varies under different conditions (Fernández-Novell *et al.*, 1992a, b, 1996, 1997; Ferrer *et al.*, 1997; Garcia-Rocha *et al.*, 2001; Cid *et al.*, 2005; Ou *et al.*, 2005; Prats *et al.*, 2005, 2009). The subcellular distribution of yeast glycogen synthase had not been addressed in any detail, being limited to data generated in a high-throughput study (Huh *et al.*, 2003). Recently, one of us (W.A.W.), addressed the localization of glycogen synthase within yeast cells through the expression of fluorescently tagged Gsy2p. It was determined that the subcellular distribution of glycogen synthase varied as a function of the glycogen content (W.A. Wilson, unpublished data). In cells that contained high levels of glycogen, Gsy2p was distributed throughout the cytoplasm. In cells that had a reduced glycogen content, Gsy2p localized to discrete regions of the cytoplasm. Finally, in cells that contained no glycogen whatsoever, a portion of the Gsy2p entered the nucleus. This was similar to the situation observed by Guinovart's group when considering the distribution of glycogen synthase in skeletal muscle cells (Ferrer *et al.*, 1997; Cid *et al.*, 2005). These workers found a nuclear uptake of glycogen synthase when glycogen was depleted (Ferrer *et al.*, 1997; Cid *et al.*, 2005).

The subcellular localization of glycogen phosphorylase was also determined in yeast, again using fluorescently labeled constructs (W.A. Wilson, unpublished data). Gsy2p and Gph1p were found to have an overlapping distribution, except under conditions where glycogen was absent. Here, while Gsy2p entered the nucleus, Gph1p adopted a diffuse cytoplasmic distribution and was excluded from the nucleus (W.A. Wilson, unpublished data). There is no evidence for a direct, physical interaction between Gsy2p and Gph1p, but both proteins are known to associate with glycogen particles (Francois & Parrou, 2001; Roach *et al.*, 2001). Our current working model holds that the distribution of glycogen synthase and glycogen phosphorylase within yeast cells may act as a marker of the distribution of glycogen particles, to which both proteins are tethered. In cells with high levels of glycogen, the diffuse distribution of glycogen synthase would be reflective of the abundance of glycogen particles distributed throughout the cytoplasm. When glycogen was absent, the glycogen synthase and phosphorylase would lose this cytoplasmic anchor. In the case of glycogen synthase, a fraction of the protein then enters the nucleus.

What might Gsy2p do within the nucleus? Glycogen serves as a store of both carbon and energy. The freeing of glycogen synthase from its cytoplasmic tether to the glycogen particle as these stores reduced would therefore be a signal that carbon and energy reserves were low. The uptake of glycogen synthase into the nucleus might therefore represent a form of molecular 'fuel gauge.' It is possible that glycogen synthase could regulate transcription in response to energy availability by some as yet undetermined means.

Future perspectives

Glycogen storage by yeast was first described over a century ago and has been the subject of quite intensive study ever since. Despite decades of effort, and considerable progress, many questions still remain. We have made the best headway in understanding the transcriptional and post-translational regulation of the enzymes of glycogen storage. However, we must complete the characterization of the transcription factors involved in the regulation of both glycogen synthase and glycogen phosphorylase. Additionally, we still have to identify the protein kinase(s) responsible for the phosphorylation and activation of glycogen phosphorylase, as well as the additional kinase(s) acting upon glycogen synthase. Studies of the Gsy1p isoform of glycogen synthase are also still in their infancy and the potential differences in the regulation between this isoform and the major Gsy2p isoform are worthy of further investigation.

Our new appreciation of the role of the vacuole in glycogen storage opens up additional areas of study because it is now apparent that there are two, spatially distinct, pools of glycogen within yeast cells. Major questions arising from this observation include determining the size of the vacuolar pool in relation to the total cellular glycogen content, and ascertaining whether this pool is regulated differently from the overall glycogen levels that have traditionally been analyzed. In addition to consideration of the subcellular distribution of glycogen pools, the subcellular distribution

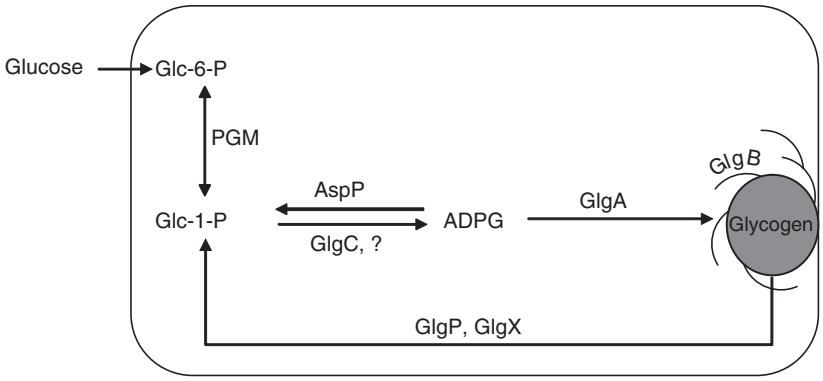

Fig. 7. Schematic model of glycogen metabolism in *Escherichia coli*. Glucose is incorporated into the cell and transformed stepwise to glucose-6-P, glucose-1-phosphate, ADPG and glycogen. This model involves the coupled reactions of PGM, GlgC and other (?) ADPG sources (Eydallin *et al.*, 2007a; Morán-Zorzano *et al.*, 2007a), GlgA and GlgB. Glycogen catabolism is controlled by both GlgP and GlgX. AspP prevents glycogen biosynthesis by diverting carbon flux from ADPG to other metabolic pathways.

of glycogen synthase must also now be considered because it appears that this enzyme may enter the nucleus when glycogen reserves are depleted. The mechanism of nuclear uptake and its relevance remain to be determined.

Arguably, the greatest challenge in our understanding of yeast glycogen storage is, however, determining precisely how information regarding the levels of extracellular nutrients is coupled to the regulation of the various signaling pathways (PKA, Snf1p, Pho85p and TOR) that control glycogen storage. Additionally, the various levels of crosstalk between these central signaling pathways must be defined.

The regulation of bacterial glycogen metabolism

Enzymology and spatial aspects of the process

The enzymology of the glycogen biosynthetic and degradative processes is highly conserved in most bacterial species (Ballicora *et al.*, 2003; Preiss, 2009). Figure 7 schematically represents the pathway of extracellular carbohydrate–glycogen conversion in *E. coli*. Extracellular glucose is taken up and converted into glucose-6-P by the carbohydrate phosphotransferase system (PTS). Phosphoglucomutase (PGM) then converts glucose-6-P to glucose-1-phosphate, which, in the presence of Mg^{2+} and ATP, is converted into ADP-glucose (ADPG) and inorganic pyrophosphate by means of ADPG pyrophosphorylase (GlgC) (Ballicora *et al.*, 2003). Using ADPG as the sugar donor nucleotide, bacterial glycogen is produced by the action of glycogen synthase (GlgA). The only known exception to this rule is *Prevotella bryantii* [a Gram-negative ruminal bacterium that lacks GlgC, and whose GlgA exclusively recognizes UDPG as a glucosyl donor (Lou *et al.*, 1997)]. After chain elongation by GlgA, glycogen branching enzyme (GlgB) catalyzes the formation of branched oligosaccharide chains having α-1,6-glucosidic linkages. This occurs in two phases. First, GlgB cleaves a six to nine glucosyl units long oligosaccharide from a nonreducing end of a glycogen molecule and then transfers the cleaved oligosaccharide to a C-6 hydroxyl group of a glucose residue in another part of the glycogen molecule (Preiss, 2009). Unlike yeast and mammalian cells where glycogenin participates in the initiation of glycogen synthesis, no glycogenin analog has been described in bacteria, and several fully sequenced genomes of bacteria known to accumulate glycogen have failed to reveal the presence of glycogenin homologues. Moreover, Ugalde *et al.* (2003) provided strong evidence that bacterial GlgA can not only elongate α(1,4)-linked glucans, but can also form the primer required for the elongation process by catalyzing its own glucosylation.

Genetic evidence that PTS and PGM are involved in the conversion process from extracellular glucose to internal glycogen has been obtained with *E. coli* mutants deleted in either *pts* or *pgm*, both mutants displaying glycogenless phenotypes (Eydallin *et al.*, 2007b; Montero *et al.*, 2009 and references contained therein). Genetic evidence that GlgC is the sole enzyme catalyzing the production of ADPG has been obtained with *glgC*$^{-}$ mutants such as the *E. coli* AC70R1-504 strain (Leung *et al.*, 1986; Ballicora *et al.*, 2003). This mutant displays an apparent glycogenless phenotype when exposed to iodine vapors. However, recent determinations of glycogen contents using more sensitive procedures have shown that AC70R1-504 cells, as well as other mutants totally lacking the GlgC function, can accumulate substantial amounts of glycogen (Eydallin *et al.*, 2007a; Morán-Zorzano *et al.*, 2007a). These findings add to the growing evidence of the occurrence of various important, but still unidentified sources of ADPG linked to glycogen biosynthesis in different bacterial species (Martin *et al.*, 1997; Sambou *et al.*, 2008). In this context, the identification of a trehalose glucosyltransferase (TreT) that catalyzes the reversible conversion of trehalose and ADP into ADPG and glucose in the archeon *Thermococcus litoralis* is noteworthy (Qu *et al.*, 2004). In any case, experimental work is still necessary to determine whether this novel enzymatic activity is actually involved in glycogen biosynthesis in this organism.

Glycogen metabolism is subjected to allosteric regulation of enzymes (Deutscher *et al.*, 2006; Preiss, 2009). In general, GlgC activators in heterotrophic bacteria are key metabolites

that represent signals of high carbon and energy contents within the cell, whereas inhibitors of this enzyme are intermediates of low metabolic energy levels. Exceptions to this rule are GlgCs from different *Bacillus* spp., as they are apparently unregulated enzymes (Ballicora *et al.*, 2003). In the case of *E. coli*, fructose-1,6-bisphosphate activates GlgC, whereas AMP acts as an important inhibitor. In support of the important role of GlgC allosteric modulation in the regulatory aspects of glycogen metabolism, *E. coli* cells bearing a mutated GlgC form that is insensitive to AMP allosteric modulation (Govons *et al.*, 1973), and *purA*$^{-}$ cells impaired in the first committed step of AMP biosynthesis (Montero *et al.*, 2009), both display glycogen-excess phenotypes. Allosteric regulation of GlgC has been extensively reviewed in recent works also including aspects relating to the structure–function relationships of GlgC, GlgA and GlgB (Ballicora *et al.*, 2003; Preiss, 2009), to which readers are referred for further information.

Glycogen phosphorylase (GlgP) (which removes glucose units from the nonreducing ends of the glycogen molecule) and debranching enzyme (GlgX) participate in the degradation of glycogen during extended periods of substrate deprivation (Dauvillée *et al.*, 2005; Alonso-Casajús *et al.*, 2006) (Fig. 7). A strong and highly specific interaction between *E. coli* GlgP and the PTS component HPr was found by surface plasmon resonance ligand fishing (Deutscher *et al.*, 2006). GlgP binding to HPr is maximal when HPr is totally phosphorylated. Furthermore, GlgP activity is increased when it binds to unphosphorylated HPr. Because the cellular concentration of HPr is much higher than that of GlgP, it has been proposed that GlgP activity is regulated by the phosphorylation status of Hpr, therefore allowing the accumulation of glycogen at the onset of the stationary phase under glucose-excess conditions (Deutscher *et al.*, 2006).

Adenosine diphosphate sugar pyrophosphatase (AspP) catalyzes the hydrolytic breakdown of ADPG linked to glycogen biosynthesis (Moreno-Bruna *et al.*, 2001). Its activity is positively affected by both glucose-1,6-bisphosphate and nucleotide–sugars and also by macromolecular crowding (Morán-Zorzano *et al.*, 2007b). Increased macromolecular crowding can affect the activity, assembly status, complex formation and binding to the cell structures of many enzymes, and becomes more pronounced as cells enter the stationary phase (Makinoshima *et al.*, 2003). This strongly suggests that AspP functions are also tightly regulated in the bacterial cell, likely playing a relevant role in preventing ADPG accumulation and in diverting the carbon flux from glycogen biosynthesis to other metabolic pathways in response to biochemical needs (Morán-Zorzano *et al.*, 2008).

Glycogen granules and several enzymes involved in bacterial glycogen metabolism are localized in the cell periphery (Chambost *et al.*, 1973; Pulkownik & Walker, 1976; Cattanéo *et al.*, 1979; Spatafora *et al.*, 1995; Makinoshima *et al.*, 2003; Eydallin *et al.*, 2007a; Morán-Zorzano *et al.*, 2008) (Fig. 8), which suggests the possible occurrence of microcompartments, wherein the enzymes are physically associated in complexes (metabolons) to facilitate metabolite channeling. Thus, changes in macromolecular crowding and translocation of glycogen metabolism-associated enzymes in response to specific extra- and intracellular signals may regulate the formation of metabolic complexes and metabolite channeling, resulting in changes in the glycogen content (Morán-Zorzano *et al.*, 2008).

Organization of structural glycogen genes

One of the salient features in bacterial gene expression is that genes of related functions are often clustered in a single operon, which ensures the simultaneous expression of functionally related gene products. A number of bacteria possess a single glycogen operon comprising all *glg* genes (Ugalde *et al.*, 1998; Kiel *et al.*, 1994; Marroquí *et al.*, 2001; Lepek *et al.*, 2002) (Fig. 9). However, it is widely accepted that genes involved in *E. coli* and *Salmonella* glycogen metabolism are clustered in two tandemly arranged operons: *glgBX* (encoding proteins that control the architectural aspects of the glycogen granule) and *glgCAP* (encoding proteins involved in the synthesis and breakdown of glycogen) (Preiss, 2009). The main evidence supporting this hypothesis comes from *in vitro* analyses showing that, unlike *glgB*, *E. coli glgC* and *glgA* expression is enhanced by the cAMP/cAMP receptor protein (CRP) complex and by guanosine 5′-(tri)diphosphate 3′-diphosphate (p)ppGpp (Romeo & Preiss, 1989). These observations, however, conflict with recent transcriptomic analyses showing that the expression of the five *glg* genes (*glgB*, *glgX*, *glgC*, *glgA* and *glgP*) was not affected in different *E. coli* Δ*crp* mutants lacking CRP (Gosset *et al.*, 2004; Zheng *et al.*, 2004; Hollands *et al.*, 2007) and also that all five *glg* transcript levels were downregulated to similar extents in *E. coli* Δ*relA*Δ*spoT* double mutants totally lacking (p)ppGpp production (Traxler *et al.*, 2008). S1 nuclease protection assays identified up to four different transcripts initiating within a 0.5-kbp region upstream of *glgC* (Romeo & Preiss, 1989), indicating that (1) *glgCAP* promoter sequences are located in the region immediately upstream from *glgC* and (2) *glgBX* and *glgCAP* constitute two different operons. However, no *E. coli* consensus promoter sequences could be found in the region upstream from *glgC*, which comprises the 3′end of *glgX* (Romeo & Preiss, 1989). In addition, Dauvillée *et al.* (2005) and Montero *et al.* (2009) have recently shown that mutants lacking the complete *glgX* gene display a glycogen-excess phenotype, indicating that significant levels of *glgCAP* transcription can also initiate upstream of *glgX*. Further

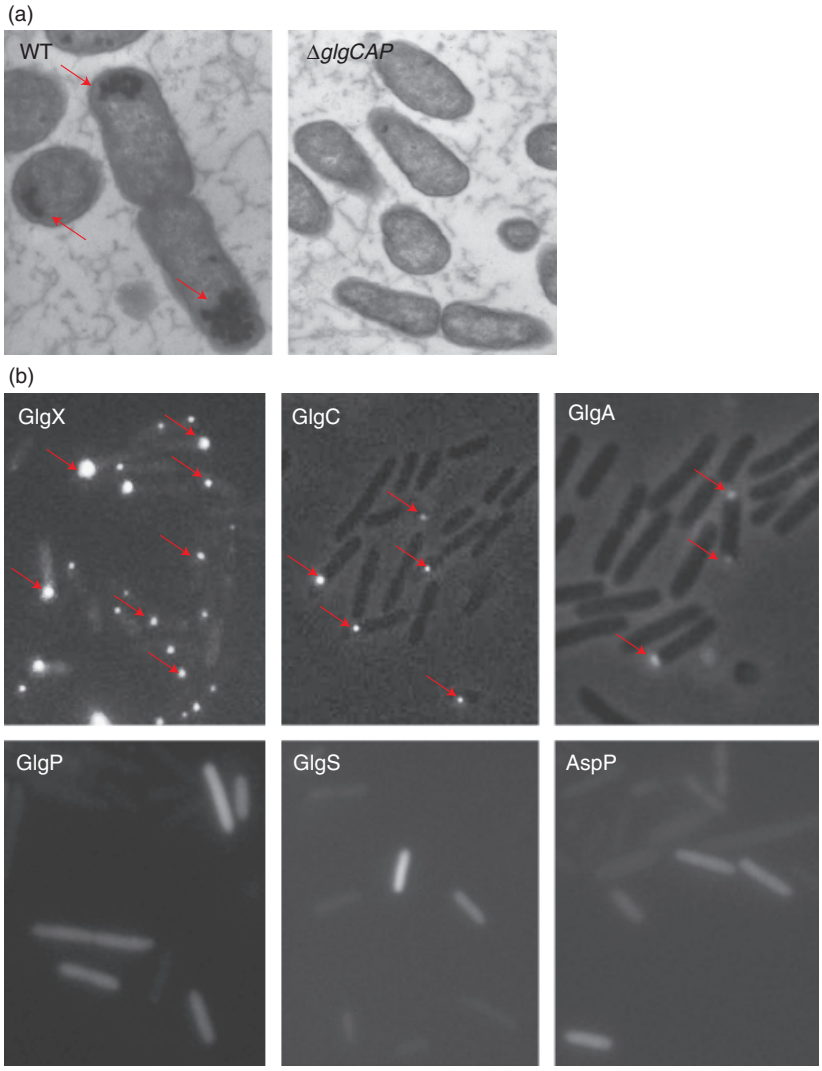

Fig. 8. Subcellular localization of glycogen granules and enzymes involved in glycogen metabolism in *Escherichia coli*. In (a), EM analysis of glycogen granules in WT and Δ*glgCAP* cells totally lacking the glycogen biosynthetic machinery. (b) Green fluorescent protein (GFP) fluorescence of cells expressing the indicated glycogen-related enzymes fused with GFP. Note that glycogen granules, GlgX, GlgA and GlgC (and also GlgB, not shown) are mainly localized in the poles of the cell (indicated with arrows), whereas GlgP, GlgS and AspP are distributed in the cytosol. Further information about distribution of GFP fluorescence in cells expressing Glg–GFP fusions can be found in http://ecoli.naist.jp/GB6/search.jsp

work is certainly needed to elucidate whether all *E. coli glgBXCAP* genes are significantly transcribed as a single mRNA unit, as well as the possible levels of regulation of the expression of these genes due to processing of this transcript.

Regulation of the expression of glycogen genes

Regulation of *E. coli* glycogen metabolism involves a complex assemblage of factors that are adjusted to the physiological and energetic status of the cell (Dietzler *et al.*, 1974; Eydallin *et al.*, 2007b; Montero *et al.*, 2009) and to cell-to-cell communication (Morán-Zorzano *et al.*, 2008). At the level of gene expression, several factors have been described to control bacterial glycogen accumulation. In *E. coli*, this includes negative regulation by the still unidentified *glgQ* regulatory locus, and by the carbon storage regulator CsrA (Romeo *et al.*, 1993; Yang *et al.*, 1996; Baker *et al.*, 2002), and positive regulation by RpoS, the PhoP/PhoQ regulatory system, the stringent response and by the cAMP/CRP complex.

RpoS is an alternative sigma factor of the RNA polymerase for the general stress response, which is required for normal glycogen biosynthesis (Lange & Hengge-Aronis, 1991; Eydallin *et al.*, 2007b, 2010; Montero *et al.*, 2009). *lacZ* fusion analyses on WT and *rpoS* mutant cells have shown that RpoS does not regulate *glgCAP* transcription in *E. coli* (Hengge-Aronis & Fischer, 1992; Montero *et al.*, 2009), but positively controls the expression of *glgS*, a gene whose product exerts a positive, but still undefined effect on glycogen accumulation (Hengge-Aronis & Fischer, 1992; Eydallin *et al.*, 2010; Montero *et al.*, 2009). This gene codes for a 7.9-kDa protein, which is hydrophilic, highly charged and has no significant sequence similarity to any other

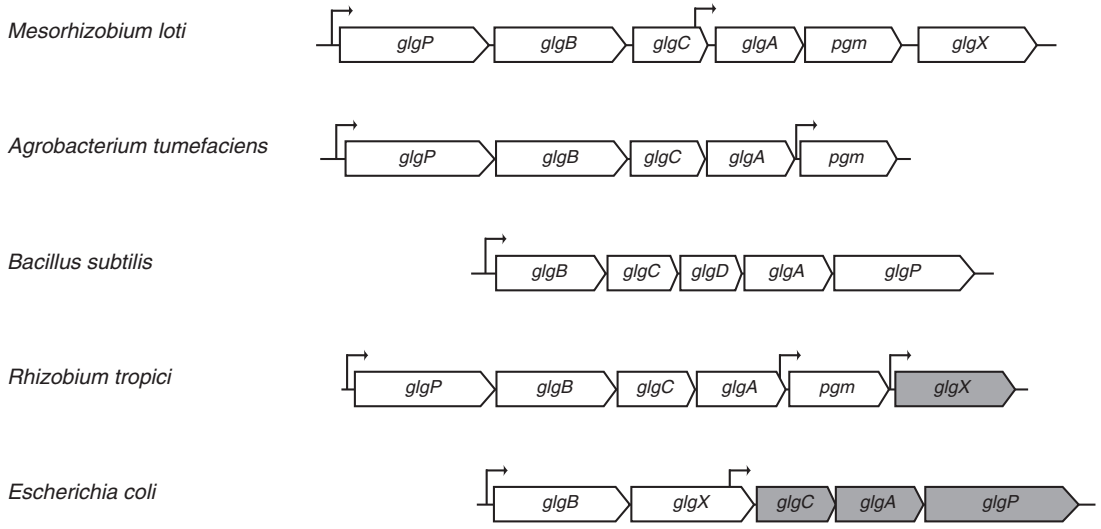

Fig. 9. Schematic representation of the known bacterial *glg* operons. Arrows indicate the position of promoter sequences. Note that *glg* operons of *Mesorhizobium loti*, *Agrobacterium tumefaciens* and *Rhizobium tropici* possess internal promoters. Structural glycogen genes in *Escherichia coli* are tandemly organized in two contiguous operons: *glgBX* (in white) and *glgCAP* (in gray).

protein present in databases outside enterobacteria (Beglova *et al.*, 1997; Kozlov *et al.*, 2004). Sequence analyses of the 1000-bp-long promoter region upstream from the ATG initiation codon of *glgS* did not reveal the presence of a putative RpoS box, defined in *E. coli* as $TGN_{0-2}CYATAMT$ (Lacour & Landini, 2004) or TCTATACTTAA (Weber *et al.*, 2005). It is thus possible that *glgS* is indirectly regulated by RpoS, as has been shown to occur in many other genes belonging to the RpoS regulon (Weber *et al.*, 2005). Intriguingly, however, recent transcriptome, proteome and *lacZ* fusion analyses failed to show that *glgS* belongs to the RpoS regulon (Lacour & Landini, 2004; Vijayakumar *et al.*, 2004; Weber *et al.*, 2005; Lelong *et al.*, 2007).

During nutrient starvation, *E. coli* elicits the so-called 'stringent response' that switches the cell from a growth-related mode to maintenance/survival mode (Dennis *et al.*, 2004; Potrykus & Cashel, 2008). The hallmark of this pleiotropic physiological response is the accumulation of the alarmones pppGpp and ppGpp (Potrykus & Cashel, 2008). While ppGpp is more abundant than pppGpp, the relative effects of these two regulatory nucleotides have not been examined thoroughly, 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 (Hara & Sy, 1983; Xiao *et al.*, 1991; Kuroda *et al.*, 1997; Potrykus & Cashel, 2008). (p)ppGpp binds bacterial RNA polymerase to increase the transcription of amino acid biosynthesis genes during amino acid starvation and to downregulate the transcription of 'stable' RNAs (rRNAs and tRNAs) genes (Dennis *et al.*, 2004; Potrykus & Cashel, 2008). As transcription of genes coding for components of the translation apparatus accounts for a large percentage of transcription in exponentially growing cells, the release of RNA polymerase from these genes is thought to passively allow upregulation of diverse promoters activated at the onset of the stationary phase (Barker *et al.*, 2001). In this respect, different *in vivo* and *in vitro* experimental evidence has linked the *E. coli* stringent response and (p)ppGpp accumulation to increased glycogen content and enhanced expression of *glg* genes at the onset of the stationary phase (Bridger & Paranchych, 1978; Taguchi *et al.*, 1980; Romeo *et al.*, 1990; Traxler *et al.*, 2008). Consistent with the involvement of (p)ppGpp in the regulatory aspects of glycogen metabolism, and also consistent with the assigned functions of SpoT and Gpp in (p)ppGpp degradation, Eydallin *et al.* (2007b) and Montero *et al.* (2009) have recently shown that *E. coli* cells impaired in the *relA* function display a glycogen-deficient phenotype as a consequence of the downregulation of glycogen gene expression. In addition, Eydallin *et al.* (2010) have shown that both *spoT* and *gpp* overexpressing cells display glycogen-deficient phenotypes. Data relating to the possible involvement of the stringent response in *glgS* expression are contradictory: whereas transcriptome analyses have recently shown that *glgS* is positively regulated by ppGpp (Traxler *et al.*, 2008), similar types of analyses failed to detect *glgS* as a member of the RelA regulon (Durfee *et al.*, 2008).

PhoP–PhoQ is a two-component regulatory system occurring in *E. coli* and *Salmonella* spp. that monitors the availability of extracellular Mg^{2+}, transcriptionally controlling the expression of many genes (García-Vescovi *et al.*, 1996). Mg^{2+} is a stabilizing factor for membranes, tRNA, ribosomes, etc. that strongly determines cell metabolic and energetic status. In fact, changes in the external Mg^{2+}

concentrations in the submillimolar range have profound effects on the ability of *E. coli* to accumulate glycogen (Montero *et al.*, 2009). Furthermore, *phoP* and *phoQ* mutants display glycogen-deficient phenotypes when cultured under conditions of limiting Mg^{2+} concentration (Montero *et al.*, 2009). Analyses of β-galactosidase activity levels derived from *glgC*::*lacZY* transcriptional fusions have shown that, under conditions of limiting Mg^{2+} concentration, *glgC* expression is significantly lower in cells lacking the *phoP* and *phoQ* functions than in WT cells (Montero *et al.*, 2009). Moreover, supplementation of the culture medium with Mg^{2+} largely restores *glgC*::*lacZY* expression in *phoP* and *phoQ* mutants. In addition, Western blot analyses of GlgC revealed that both *phoP* cells and *phoQ* mutants accumulated lower levels of this protein than WT cells when cultured in the presence of low Mg^{2+}, the overall data indicating that the expression of the *E. coli glg* genes is under control of the PhoP–PhoQ system under low environmental Mg^{2+} conditions (Montero *et al.*, 2009). Noteworthy, sequence analyses of the 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) or (T/G)GTTTA tandem direct repeats (Yamamoto *et al.*, 2002; Minagawa *et al.*, 2003). It is thus possible that the PhoP–PhoQ-mediated Mg^{2+} regulation of *glgCAP* is indirect.

CRP is one of the best-known global regulatory proteins in *E. coli*. cAMP produced by the membrane-bound adenylate cyclase (the product of *cya*) has been proposed to be a sensory signal in global gene control that acts through CRP (Kolb *et al.*, 1993). Variations in the level of cAMP/CRP complex in response to the presence of extracellular glucose contribute to the fine regulation of several operons. Different studies have shown that *cya* and *crp* mutants display marked glycogen-deficient phenotypes (Leckie *et al.*, 1983; Montero *et al.*, 2009), which indicates that cAMP complexed to CRP (1) is required for normal glycogen accumulation and (2) serves as an important regulator of the transcription of genes involved in glycogen metabolism. cAMP/CRP is required for the expression of *glgS* and PTS-related genes (Hengge-Aronis & Fischer, 1992; Gosset *et al.*, 2004), which in turn are required for normal glycogen production. As to the possible regulation of glycogen structural genes by the *cya* and *crp* gene products, *in vitro* experiments have shown that cAMP/CRP positively regulates the expression of *E. coli glgC* and *glgA*, but not that of *glgB* (Urbanowski *et al.*, 1983; Romeo & Preiss, 1989; Romeo *et al.*, 1990). However, recent transcriptome analyses failed to indicate that *E. coli glg* genes belong to the CRP regulon (Gosset *et al.*, 2004; Zheng *et al.*, 2004). Furthermore, using a chromosomal *glgC*::*lacZY* fusion constructed on a *cya* mutant, Montero *et al.* (2009) have recently shown that the expression of the *glgCAP* operon is not affected by the lack of cAMP production. The overall data thus indicate that the control of *E. coli* glycogen metabolism by cAMP/CRP may be due, at least in part, to the positive effect of this cyclic nucleotide on the expression of PTS-related genes rather than a direct effect on the expression of glycogen genes.

Glycogen metabolism is highly interconnected with a wide variety of cellular processes

Using the Keio collection of gene-disrupted mutants of *E. coli* (Baba *et al.*, 2006) and the ASKA gene expression library (Kitagawa *et al.*, 2005), studies have been recently carried out aimed to uncover the mechanisms regulating glycogen metabolism and its connection with other biological processes in *E. coli* (Eydallin *et al.*, 2007b, 2010; Montero *et al.*, 2009). These studies have revealed that bacterial glycogen metabolism is highly interconnected with a wide variety of cellular processes, being affected by proteins that can be placed within the following groups: (1) stringent response, (2) general stress response, (3) low extracellular Mg^{2+} availability, (4) carbon sensing, transport and metabolism, (5) factors determining intercellular communication, aggregative and social behavior, (6) sulfur metabolism, (7) nitrogen metabolism, (8) iron metabolism, (9) end-turnover of tRNA, (10) envelope composition and integrity, (11) energy production and cellular redox status, (12) small RNAs (sRNAs)-binding proteins, (13) nucleotide metabolism and (14) osmotic stress (Supporting Information, Tables S1 and S2). Functional groups (1)–(4) have been discussed above. Therefore, in the following sections, we will discuss the possible link between glycogen metabolism and groups (5)–(14).

Factors determining intercellular communication, aggregative and social behavior modes

Morán-Zorzano *et al.* (2008) have recently shown that AspP binds to cell membranes as the bacterial population density increases, remaining membrane-associated as glycogen depletes during the stationary phase. This process is stimulated by small soluble molecule(s) occurring in cell-free spent supernatants of stationary cultures, thus providing a first set of evidence that glycogen metabolism may be subjected to regulation by cell-to-cell communication. 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 regulate metabolic processes co-ordinately. Communication, aggregative and social behavior 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. Noteworthy, gaining-of-function of GGDEF and EAL domain enzymes controlling the intracellular levels of cyclic di-guanosine monophosphate

(c-di-GMP) [a secondary messenger that regulates the transition from the motile, planktonic state to sessile, community-based behaviors in different bacteria (Simm *et al.*, 2004; Wolfe & Visick, 2008; Hengge, 2009)] result in changes in the intracellular glycogen content (Eydallin *et al.*, 2010). For instance, upregulation of YeaP [a diguanylate cyclase that positively regulates the expression of *csg* genes involved in curli and cellulose production (Sommerfeldt *et al.*, 2009)] exerts a negative effect on glycogen accumulation, whereas upregulation of YjcC [a predicted c-di-GMP phosphodiesterase that downregulates the expression of the CsgD central regulator of extracellular matrix components (Christen *et al.*, 2005; Römling, 2005; Simm *et al.*, 2007)] and Dos [a cyclic diguanylate phosphodiesterase (Schmidt *et al.*, 2005)] results in enhanced glycogen content.

Nitrogen and sulfur metabolism

It is known that carbon metabolism is subjected 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 (Reizer *et al.*, 1992). Consistently, *ptsN* overexpressing *E. coli* cells are characterized by a marked glycogen-deficient phenotype when cultured in glucose Kornberg medium (Eydallin *et al.*, 2010).

Yeast extract (the amino acid source of the Kornberg medium) is deficient in amino acids such as cysteine (Reitzer, 1996; Eydallin *et al.*, 2007b). Mutants impaired in functions involved in cysteine biosynthesis display a glycogen-excess phenotype when they are cultured in Kornberg medium (Montero *et al.*, 2009), which can be ascribed to the stringent response-mediated upregulation of *glg* genes due to the lack of cysteine provision (Eydallin *et al.*, 2007b). Confirming this view, these mutants display a normal glycogen content phenotype when Kornberg medium is supplemented with cysteine. 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.

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 a nutritional downshift, which is a process involving a major portion of the maintenance energy requirement (Gottesman, 2003). Cells impaired in *lon*, *clpP* or *clpA* display a glycogen-excess phenotype, which is ascribed to (1) deviation of energy flux from protein degradation to glycogen biosynthesis and (2) elicitation of the stringent response due to the lack of internal amino acid provision (Eydallin *et al.*, 2007b; Montero *et al.*, 2009), which, as discussed above, leads to enhancement of the glycogen content.

Iron metabolism

Under aerobic conditions, *E. coli* utilizes high-affinity extracellular siderophores that solubilize and capture Fe(III) before transport and metabolism. Fur, a dominant sensor of iron availability, generally represses iron siderophore biosynthetic and transport genes such as *fepB*, *fepD* and *fepG* (McHugh *et al.*, 2003). Under iron-limiting conditions, iron dissociates from Fur, and increased transcription of genes ensues. Iron limitation causes the SpoT-dependent stringent response (Vinella *et al.*, 2005), which, as discussed above, leads to enhancement of the glycogen content. Consistently, *fepB*, *fepD* and *fepG* mutants display glycogen-excess phenotypes, whereas *fur* mutants display a glycogen-deficient phenotype (Montero *et al.*, 2009).

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 *rnt*) 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* (Deutscher *et al.*, 1985). *rnt* mutants impaired in tRNA turnover accumulate defective tRNA molecules and high levels of ppGpp (Deutscher *et al.*, 1977), thus resulting in increased glycogen content (Montero *et al.*, 2009).

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 antisigma RseA factor (Alba & Gross, 2004). When *E. coli* is subjected to extracytoplasmic stresses, RseA degrades and RpoE activity is induced. Another major point of regulation of RpoE takes place at the post-transcriptional level, because it has been shown that Hfq interaction with *rseA* mRNA downregulates *rseA* expression (Ding *et al.*, 2004). Noteworthy, cells impaired in RseA and Hfq functions accumulate low glycogen levels, suggesting that RpoE-mediated envelope stress response may to some extent negatively affect glycogen accumulation (Montero *et al.*, 2009). 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*, display glycogen-deficient phenotypes (Montero *et al.*, 2009). All these mutants are likely to promote both envelope stress membrane deformation that causes inhibition of the electron transport chain,

energy production and formation of membrane potential necessary for nutrient import (Andersen & Koeppe, 2007). The *rfaE* mutant for instance lacks an enzyme required for *E. coli* lipopolysaccharide biosynthesis (Valvano *et al.*, 2000). Mutants impaired in the GalU function lack the enzyme that catalyzes the synthesis of UDPG necessary for the synthesis of cell envelope components (Genevaux *et al.*, 1999). Moreover, *tolB*, *tolR*, *tolQ* and *pal* mutants do not possess proteins of the Tol–Pal system essential in maintaining envelope integrity (Lloubès *et al.*, 2001). In addition, *ponB* (*mrcB*) mutants lack a bifunctional membrane-bound enzyme catalyzing transglycosylation and transpeptidation reactions, which are essential in the late stages of peptidoglycan biosynthesis (Plá *et al.*, 1990).

Energy production and redox status

ATP is a primary signal in regulating glycogen biosynthesis, and acts as substrate for the ADPG-producing reaction catalyzed by GlgC (Preiss, 2009). Consistently, mutations in components required for the proper functioning of the aerobic electron transport chain and ATP generation negatively affect glycogen accumulation (Eydallin *et al.*, 2007b; Montero *et al.*, 2009). Thus, *ubiG* and *ubiH* mutants (deficient in ubiquinone production), *iscU*, *iscS*, *fdx* and *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), and *gshB* and *gor* mutants (lacking the machinery necessary to produce and reduce glutathione) showed reduced glycogen levels.

sRNAs-binding proteins

During the last decade, noncoding sRNAs have been shown to be involved in gene regulation. In prokaryotes, many sRNAs are specifically expressed during adaptation to nutritional stress (Gottesman, 2004). To date, close to 100 different sRNAs have been identified in *E. coli*, many of them regulating target mRNAs at the post-transcriptional level via direct base pairing, and, as a consequence, modifying translation and/or message stability. The majority of these sRNAs require binding proteins such as Hfq and CsrA for proper function in gene regulation.

The global regulator CsrA is a RNA-binding protein controlling a wide variety of biological processes, such as glycogen synthesis, glycolysis, motility and biofilm formation. It prevents glycogen accumulation by both promoting *glgCAP* decay and preventing *glgC* translation (Liu *et al.*, 1995; Baker *et al.*, 2002). Furthermore, CsrA negatively affects the expression of *glgS* (Yang *et al.*, 1996) and *hfq* (Baker *et al.*, 2007; Dong & Schellhorn, 2009). CsrA activity is antagonized by the two CsrB- and CsrC-noncoding sRNAs (Liu *et al.*, 1997; Weilbacher *et al.*, 2003; Dubey *et al.*, 2005), which in turn are targeted by CsrD for RNase E degradation (Suzuki *et al.*, 2006). Consistent with the negative effect of CsrA in glycogen accumulation, and consistent with the assigned role of CsrD as relieving CsrA function from CsrB and CsrC in *E. coli*, recent studies have shown that *csrA* and *csrD* overexpressing *E. coli cells* display a glycogen-deficient phenotype (Eydallin *et al.*, 2010). Noteworthy, and unlike the situation in *E. coli*, CsrA does not apparently play a relevant role in the regulation of glycogen accumulation in *Salmonella enterica* (Lawhon *et al.*, 2003).

Hfq is a chaperone that stabilizes many regulatory sRNAs and facilitates the base pairing between sRNAs and their target mRNAs (Brennan & Link, 2007). The cellular role of the main part of the Hfq-binding sRNAs is to control stress responses. Deletion of *hfq* has pleiotropic phenotypes, including slow growth, altered cell division, osmosensitivity, increased oxidation of carbon sources, deficiency in the σ^E-mediated general stress response, accumulation of outer membrane proteins and altered patterns of protein synthesis (Valentin-Hansen *et al.*, 2004). Mutants impaired in the *hfq* function have altered levels of expression of master transcription factors, and of genes affecting glycogen accumulation such as those involved in glucose transport, cysteine metabolism, nitrogen metabolim, purine metabolism and glycogen structural genes such as *glgB*, *glgX*, *glgC* and *glgA* (Guisbert *et al.*, 2007; Sittka *et al.*, 2008). Noteworthy, these mutants also display a marked glycogen-deficient phenotype (Eydallin *et al.*, 2007b; Montero *et al.*, 2009) (1), showing that Hfq is necessary for normal glycogen accumulation and (2) suggesting the involvement of sRNAs in the regulation of *E. coli* glycogen metabolism. In fact, Hfq binds RprA and DsrA [both activators of RpoS (Majdalani *et al.*, 2001)] and OxyS [a RpoS repressor (Gottesman, 2004)]. Hfq also binds RyaA, which strongly downregulates the expression of the glucose-specific PTS transporter encoding gene *ptsG* (Vanderpool & Gottesman, 2004). Furthermore, Hfq binds RybB and MicA (Figueroa-Bossi *et al.*, 2006; Thompson *et al.*, 2007), both acting as negative regulators of RpoE, which, as discussed above, exerts a negative effect in glycogen accumulation. Noteworthy, Hfq also binds *glgS* mRNAs (Zhang *et al.*, 2003), strongly suggesting that the stability and/or the translation potential of *glgS* mRNAs is affected by Hfq.

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

Figure 10 illustrates a suggested model of glycogen metabolism in *E. coli* wherein major determinants of glycogen accumulation include levels of intracellular Mg-bound ATP necessary for ADPG synthesis [determined by extracellular

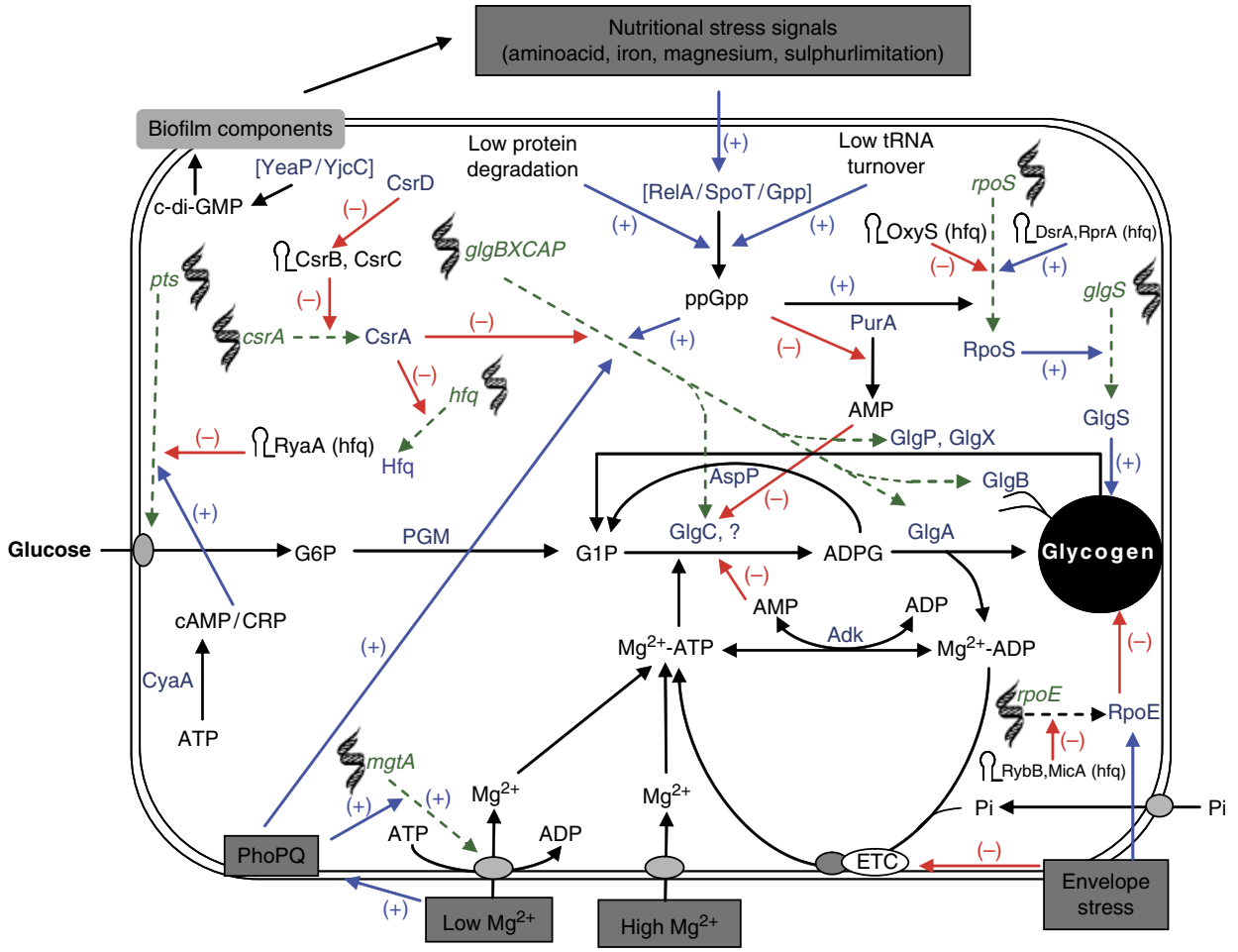

Fig. 10. Suggested integrated model of glycogen metabolism. Suggested metabolic model wherein major determinants of glycogen metabolism include the PhoP–PhoQ regulatory system activated under low extracellular Mg^{2+} concentration conditions, intracellular concentration of Mg-bound ATP necessary for ADPG synthesis [determined by extracellular Mg^{2+} concentration, transport of Mg^{2+} across membranes, ATP synthesis and consumption, and adenylate kinase (Adk) activity], levels of AMP (the main GlgC inhibitor and indicator of the low energetic status of the cell), 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, sulfur, iron, etc.), factors determining intercellular communication, aggregative and social behavior modes (which in turn determine the nutritional status of the cell), levels of cAMP, expression levels of the general stress regulator RpoS and of the global regulators CsrA and Hfq, 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 (ETC). sRNAs, represented by stem-loops, are likely involved in the regulation of functions strongly affecting glycogen accumulation through interaction with CsrA and Hfq. 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 a carbon source is present in the medium.

Mg^{2+} concentrations, transport of Mg^{2+} across membranes, ATP synthesis and consumption and adenylate kinase (Adk) activity (Igamberdiev & Kleczkowski, 2003)], levels of AMP (the main GlgC inhibitor and indicator of the low energetic status of the cell), 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, sulfur, Mg^{2+}, iron, etc.), factors determining intercellular communication, aggregative and social behavior modes (which in turn determine the nutritional status of the cell), expression levels of the general stress regulator RpoS and of the global regulators CsrA and Hfq, 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. sRNAs are likely involved in the regulation of functions directly or indirectly affecting glycogen accumulation through interaction with Hfq. 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 toward glycogen biosynthesis when an excess carbon source is present in the medium.

Research needs

Over the past 5–10 years, improvements have been achieved in understanding how bacterial glycogen is regulated at the molecular level and how it is connected with other biological processes. A substantial part of this advance has been due to the availability of systematic and comprehensive gene disruption (Keio) and expression (ASKA) collections. However, considerable important information is still missing before a comprehensive understanding of the entire process can be attained. With emerging new techniques, the next decade will undoubtedly give us a better understanding of features, which, at present, are not completely understood such as the involvement of sRNAs in the regulation of carbohydrate metabolism in general and of glycogen in particular. In the short term, the identification and characterization of sources, other than GlgC, of ADPG (or any other glucosyl donor) linked to glycogen biosynthesis constitutes one of the major challenging objectives in the field of bacterial glycogen metabolism. Because glycogen appears to play relevant roles in the survival of bacteria to sporadic periods of famine, and because glycogen metabolism is highly interconnected with multiple and important cellular processes, it is tempting to speculate that redundancy of ADPG sources could have been selected during bacterial evolution to ensure the production of glycogen.

The precise molecular function of GlgS is still poorly resolved. This protein is important in the glycogen biosynthetic process, because it stimulates the synthesis of this polyglucan when overexpressed in the cell (Hengge-Aronis & Fischer, 1992; Eydallin *et al.*, 2010). Earlier work suggested that GlgS might be a site for primary sugar attachment during the glycogen initiation process (Beglova *et al.*, 1997). However, this hypothesis has been weakened by the finding that *Agrobacterium tumefaciens* GlgA does not require additional proteins for glycogen priming (Ugalde *et al.*, 2003). The GlgS structure, suggesting a role in protein–protein interactions, offers alternative possibilities for its function (Kozlov *et al.*, 2004). The stimulation of glycogen synthesis by GlgS overproduction may be caused by GlgS-mediated interactions between proteins involved in glycogen biosynthesis. As discussed above, changes in translocation of glycogen enzymes in response to specific extra- and intracellular signals would regulate the formation of metabolic complexes and metabolite channeling, which in turn would regulate glycogen metabolism. Protein–protein interaction analyses of GlgS would help to test this hypothesis. Noteworthy, protein complexes facilitating functional interactions among starch/glycogen metabolic enzymes have been recently shown to occur in plants (Tetlow *et al.*, 2008; Hennen-Bierwagen *et al.*, 2009).

As discussed by Montero *et al.* (2009), monitoring nutritional status is essential in optimizing bacterial survival strategies, including glycogen accumulation and/or breakdown. cAMP, (p)ppGpp and c-di-GMP are among the most comprehensively studied nucleotide-based second messengers. Although both cAMP and (p)ppGpp have long been linked to regulatory aspects of glycogen metabolism, only recently evidences have been provided suggesting the possible occurrence of c-di-GMP-mediated mechanisms regulating glycogen accumulation (Eydallin *et al.*, 2010). c-di-GMP is an alarmone that is used by most bacteria to orchestrate the switch between free-living and sedentary life styles, controlling numerous cellular functions such as the biosynthesis of adhesions and exopolysaccharide matrix components, long-term survival and response to environmental stresses, synthesis of secondary metabolites, regulated proteolysis and cell cycle progression, etc. (Schirmer & Jenal, 2009). More work is needed to investigate the possible involvement of c-di-GMP in glycogen metabolism regulation and connection with other biological processes.

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Authors' contribution

W.A.W. and M.M. contributed equally as first authors to this work on the yeast and bacterial sections, respectively.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of genes (and functions of their products) whose ectopic expression affects glycogen content (Eydallin *et al*., 2010).

Table S2. List of genes (and functions of their products) whose deletion affects glycogen content (Eydallin *et al*., 2007b; Montero *et al*., 2009).

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