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Abstract In eukaryotic cells, glycogenin is a self-glucosylating protein that primes glycogen synthesis. In yeast, the loss of function of *GLG1* and *GLG2*, which encode glycogenin, normally leads to the inability of cells to synthesize glycogen. In this report, we show that a small fraction of colonies from *glg1glg2* mutants can switch on glycogen synthesis to levels comparable to wild-type strain. The occurrence of glycogen positive *glg1glg2* colonies is strongly enhanced by the presence of a hyperactive glycogen synthase and increased even more upon deletion of *TPS1*. In all cases, this phenotype is reversible, indicating the stochastic nature of this synthesis, which is furthermore illustrated by colour-sectoring of colonies upon iodine-staining. Altogether, these data suggest that glycogen synthesis in the absence of glycogenin relies on a combination of several factors, including an activated glycogen synthase and as yet unknown alternative primers whose synthesis and/or distribution may be controlled by *TPS1* or under epigenetic silencing.

Keywords: Glycogenin; Glycogen; Trehalose; Glycogen synthase; *GSY2*; *TPS1*

1. Introduction

Glycogen is a polymer of glucosyl units linked by α-1,4-bonds with α-1,6-branches. It can accumulate to account for up to 10–15% of the cell dry mass in yeast under conditions of growth restriction, upon specific physicochemical stresses and at the end of growth on a glucose-limited medium [1]. In eucaryotic cells, the biogenesis of glycogen is initiated by glycogenin, a self-autoglucosylating protein that produces, from UDP-glucose, a short oligosaccharide covalently linked to a tyrosine residue of this initiator protein. Once the oligosaccharide chain has been extended sufficiently (6–10 glucose residues), glycogen synthase catalyzes the elongation and, together with the action of a branching enzyme, generates a mature glycogen molecule of very high molecular mass [2,3]. In the yeast *Saccharomyces cerevisiae*, *GLG1* and *GLG2* encode glycogenin-like proteins that are 55% identical to each other and 33% identical to the rabbit muscle glycogenin [4]. Disruption of either gene causes no defect in glycogen accumulation, but deletion of both genes was shown to abolish glycogen synthesis. The same result was recently obtained by disrupting the *gnn* gene that encodes the *Neurospora crassa* glycogenin [5]. Taken together, these genetic data are the strongest proof to date that a protein primer is necessary for glycogen biogenesis in eucaryotic cells.

Until now, no glycogenin-like protein has been found in bacteria [6], which suggests that the initiation of this polymer must occur in a different way. Ugalde et al. [7] recently showed that the de novo synthesis of glycogen in *Agrobacterium tumefaciens* is initiated directly on glycogen synthase, which catalyzes both the autoglucosylation and the elongation process. Also, in mammalian tissues, it was shown that alkylglucosides and aromatic glucosides can serve as artificial acceptors for the transfer of glucosyl unit from UDP-glucose by glycogen synthase yielding alkylmaltooligosaccharide products, which can be further elongated into α-(1,4) glucosyl chains by the same enzyme [8]. Whether such oligosaccharide acceptors exist in vivo is still an open question. There was also a report of the existence of a manganese sulfate-dependent glucose transfer to glycoproteins that is catalyzed by a non-glucose 6-phosphate-activated glycogen synthase [9], but the role of this process in the early stage of glycogen biogenesis is still obscure. Together, these data raise the question of whether glycogen biogenesis in eukaryotic cells could still occur in the absence of glycogenin. In this report, we show that glycogen synthesis can take place in glycogenin-defective strains of *S. cerevisiae*, and discuss possible mechanisms underlying this process.

2. Materials and methods

2.1. Yeast strains, plasmids and growth conditions

Construction of strains from EG3218-1A and CEN.PK113-1A background was described previously [10,11]. Unless otherwise stated, yeast strains were grown at 30 °C in a synthetic minimal medium containing 2% (w/v) galactose (YNGal) or glucose (YNGlu), 0.17% (w/v) yeast nitrogen base without amino acids and ammonium, 0.5% (w/v)

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ammonium sulfate, supplemented with the appropriate auxotrophic requirements. The same medium with only 0.02% (w/v) ammonium sulfate was prepared for nitrogen limitation experiments. Agar was added at 2% (w/v) for solid media. The *tps1* strains cannot grow on glucose. Therefore, for rigorous comparisons, galactose was routinely used. This carbon source further leads to enhanced glycogen deposition in yeast cells as compared to glucose, and makes easier identification and counting with better contrast between strains that do and do not accumulate glycogen.

Plasmids YEp356 and pYADE4 were used as 2μ control vectors carrying *URA3* and *TRP1* markers, respectively. Plasmids carrying full length *GSY2* and mutated variants in the COOH-terminal have been described previously [12,13]. These constructs will be referred in this study to as pGSY2 (pYcDE2-GSY2; 2μ, *TRP1*, *GSY2* CDS under the *ADH1* promoter), pGSY2*-CEN (pRS314-GSY2 S650A/S654A; CEN/ARS, *TRP1*, own promoter), pGSY2Δ643-2μ (pYcDE2-GSY2Δ643; 2μ, *TRP1*, *GSY2* CDS under *ADH1* promoter). The multicopy vector carrying the full length *GAC1* gene is referred to as pGAC1-1 (pST93; 2μ, *URA3* [14]) or pGAC1-2 (same as pGAC1-1 with *TRP1* as marker; unpublished).

2.2. Biochemical and analytical procedures

Yeast samples (50 OD_{600} units) were filtered through nitrocellulose membranes. The cells were rapidly scraped, frozen in liquid nitrogen and stored at −80 °C until use. Preparation of extracts and assay of glycogen synthase were carried out as described by François et al. [15] in the presence of 0.25 mM UDP [U–^{14}C] glucose. To estimate the active and total form, the assay was done in the absence and in the presence of 5 mM galactose-6-P instead of glucose-6-P because galactose-6-P can act as a glycogen synthase activator with a $K_a \cong 0.5$ mM (François, unpublished), and this avoided isotopic dilution of UDP [U–^{14}C] glucose due to the presence of active galactose-1-phosphate uridyl transferase and UDP-galactose epimerase in crude extract of galactose-grown cells.

2.3. Determination of glycogen and metabolites

Qualitative assessment of glycogen content was carried out by the iodine-staining method of Chester [16] following the modification of Enjalbert et al. [17]. Quantitative assays of glycogen and trehalose levels were performed according to Parrou and François [18]. Collection of yeast cells for extraction of intracellular metabolites and their measurement were carried out as in [19,20].

2.4. Isolation of glycogen and determination of its structure by proton nuclear magnetic resonance spectrometry (^{1}H NMR)

Glycogen was purified from wild-type and mutant strains grown on YNGal (i.e., at $OD_{600} \approx 15$). Briefly, about 0.5–1 g cells (dry mass) were disrupted in 20 ml of 50 mM sodium β-glycerophosphate buffer, pH 7.6, containing 2 mM EDTA, 2 mM EGTA and a protease inhibitor cocktail (Roche, 1836170; 1 capsule for 10 ml of buffer) with 0.5 g glass beads (0.5 mm diameter) by vigorous vortex mixing, 6 times for 15 s, with 15 s intervals on ice. The supernatant was collected by a 10 min centrifugation at 3000 × *g*, 4 °C. A second centrifugation at 15000 × *g*, 4 °C for 45 min was followed by a third ultracentrifugation of the latter supernatant at 100000 × *g*, 4 °C for 1 h 20 min. The pellet, which contained glycogen, was washed with 10 ml of extraction buffer, resuspended in 1 ml of the same buffer, and ethanol (to a final 66% v/v) was added. The glycogen pellet was collected by centrifugation (5 min at 10000 × *g* in a microfuge), rinsed with 66% cold ethanol. After removal of excess of ethanol by incubation at room temperature, the purified glycogen (about 20 mg) was resuspended in a minimal volume of 50 mM ammonium bicarbonate, pH 7.6, in the presence of 0.02 mg/ml of trypsin. The suspension was incubated at 37 °C for 5 h, then the same amount of trypsin was added and the solution was incubated for another 5 h. After digestion, samples were dialyzed overnight at 4 °C against MilliQ water (SpectraPor Membrane MWCO: 6-8000 Spectrum) and lyophilized. The samples were then analysed by Nuclear Magnetic Resonance. ^{1}H NMR analyses were performed on a Bruker AMX-500 spectrometer at 500.13 MHz using a 5 mm BBI probe at 343 °C in D_2O. COSY experiments were performed using the Bruker pulse field gradient program cosygpmf, with 1.5 s recycle delay and 0.52 s acquisition time. A sine-bell apodization function was applied before Fourier transformation.

3. Results

3.1. Yeast can synthesize glycogen in the absence of glycogenin

A very simple method to evaluate glycogen accumulation in yeast is to spot yeast cultures on agar plates and then to check whether these patches stain brown upon exposure to iodine vapour [16,17]. As indicated in Fig. 1A, the patch of the *glg1glg2* mutant remained yellow whereas the wild-type strain was brown. However, when a culture of *glg1glg2* cells was spread on YNgal agar plates to generate isolated colonies, we surprisingly found that 2–3% of these colonies were brown after iodine staining (Fig. 1B, left panel). The colour of the colonies, but not the frequency of their apparition, was considerably enhanced when the medium was nitrogen-limited (0.02% ammonium sulfate instead of 0.5%; Fig. 1B, right panel), a condition known to favour glycogen deposition [17,21]. Moreover, this phenotypic trait was associated neither with nature of the carbon source (identical results with glucose, data not shown), nor with the genetic background of the strain since similar results were obtained with the CC9 strain [4], a *glg1glgl2* mutant generated in a different genetic context (data not shown). Under this condition, 100% of the isolated colonies from wild-type strains turned brown when exposed to iodine vapour (data not shown).

To verify that the brownish colouration of *glg1glg2* mutant colonies was due to glycogen, and not to other molecules such as lipids that are known to interfere somehow with the iodine staining [22], we performed two types of experiments. In the first, brown colonies of *glg1glg2* mutant were cultivated in

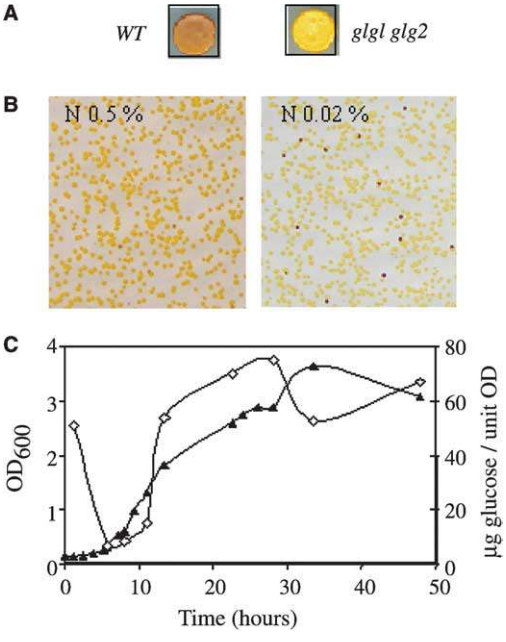

Fig. 1. (A, B) Iodine staining of a wild-type and glycogenin-defective strains. The wild-type CENPK113-1A and corresponding *glg1glg2* mutant were pre-cultured in liquid YNGal and 10 μl were spotted on a YNgal plate (A) or spread to obtain isolated colonies on galactose plates with high (left panel) or low ammonium sulfate (right panel). (C) Enzymatic determination of glycogen during growth of 'iodine-positive' glycogenin-defective colonies. An overnight pre-culture of iodine-positive colonies from a *glg1glg2* mutant in YNGal medium limited for ammonium sulfate was inoculated in 100 ml of the same medium for glycogen determination. Symbols: (▲) OD_{600}, (◇) glycogen levels.

nitrogen-limiting YNGal medium and samples from this culture were subjected to digestion by α-amylo-(1,4)–(1,6) glucosidase from *A. niger*. As shown in Fig. 1C, this culture accumulated glycogen up to 60 μg equivalent glucose per OD_{600} unit, i.e., ∼ 10% of dry mass, even though cell growth was weak due to nitrogen limitation. Glycogen metabolism in these *glg1glg2* cells was similar to what occurs in wild-type cells [21], as shown by a rapid degradation of the stored glycogen after inoculation and synthesis that started as the growth was restricted by the nitrogen limitation. In the second experiment, we analyzed the structure of glycogen by Proton Nuclear Magnetic Resonance Spectrometry (^{1}H NMR). Both 1D and 2D-COSY ^{1}H NMR-spectra of purified polysaccharides from wild-type and the *glg1glg2* mutant were superimposable on those of 'normal' glycogen [23] (data not shown).

3.2. An activated form of glycogen synthase is required for the glycogenin-independent accumulation of glycogen

Yeast cells deleted for the *GSY1* and *GSY2* genes, which encode glycogen synthase, are unable to accumulate glycogen (Fig. 2A and [24]). However, in contrast to the *glg1glg2* mutant, no brown colonies were observed upon iodine staining of either *gsy1gsy2* or *glg1glg2gsy1gsy2* mutants grown on nitrogen-limiting agar plates (data not shown). This indicates that the synthesis of glycogen in the absence of glycogenin still proceeds through glycogen synthase. Moreover, we found that a *glg1glg2* mutant transformed with a high copy number plasmid bearing either *GSY2*Δ643, which encodes a hyperactive form of glycogen synthase [12] or *GAC1*, which encodes the targeting subunit of glycogen synthase phosphatase [14], recovered the ability to accumulate glycogen (Fig. 2A). In contrast, a high copy number plasmid bearing a construct encoding the wild-type form of Gsy2p, which enables a *gsy1gsy2* mutant to re-establish glycogen synthesis, was unable to restore glycogen synthesis in the *glg1glg2* strain (Fig. 2A). This result is consistent with the fact that the activation of glycogen synthase is severely impaired in a *glg1glg2* mutant at the onset of glycogen accumulation during growth [4]. Finally, and in confirmation of previous work [11], glycogen synthesis in cells lacking glycogenin, but not in those defective in glycogen synthase, could also be restored upon deletion of *TPS1*, which encodes trehalose-6-phosphate synthase (Fig. 2A). As shown in Fig. 2B, glycogen accumulation in *glg1glg2tps1* as well as in *glg1glg2* cells bearing hyperactive glycogen synthase followed a similar profile than in the wild-type until the entrance into the stationary phase. This result prompted us to examine the effect of *TPS1* deletion on glycogen synthase. As shown in Table 1, the activated form of the glycogen synthase was 2-fold increased upon *TPS1* disruption. The intracellular levels of UDP-glucose, the substrate of glycogen synthase, and of glucose-6-P, a potent activator of this enzyme [25], were also 1.3–2-fold higher in the *glg1glg2tps1* mutant than in the *glg1glg2* strain. Thus, the combination of a partially activated form of glycogen synthase and greater availability of UDP-glucose and glucose-6-P may explain in part the strong potency of a *glg1glg2tps1* strain to accumulate glycogen.

3.3. Evidence for a stochastic 'on–off' synthesis of glycogen in the absence of glycogenin

As shown in Fig. 3, the percentage of colonies that stained brown with iodine vapour increased from 3% in a *glg1glg2* mutant to 99% upon deletion of *TPS1* in this strain. These results raised the question as to whether colonies from *glg1glg2* mutants that stained brown had definitively acquired this phenotype, which would be consistent with a genetic reversion of the original phenotype. To verify this hypothesis, we examined the stability of the iodine-staining phenotype following successive spreading of colonies on nitrogen-limiting plates. The percentage of yellow and brown colonies from the *glg1glg2* mutant was then scored from a total of about 500 colonies after each spreading (Fig. 3A). When a brown colony was spread again on a new agar plate, about 25% of isolated cells yielded colonies that did not stain upon iodine vapour. When one of these yellow colonies was spread again on YNGal, less than 0.5% of

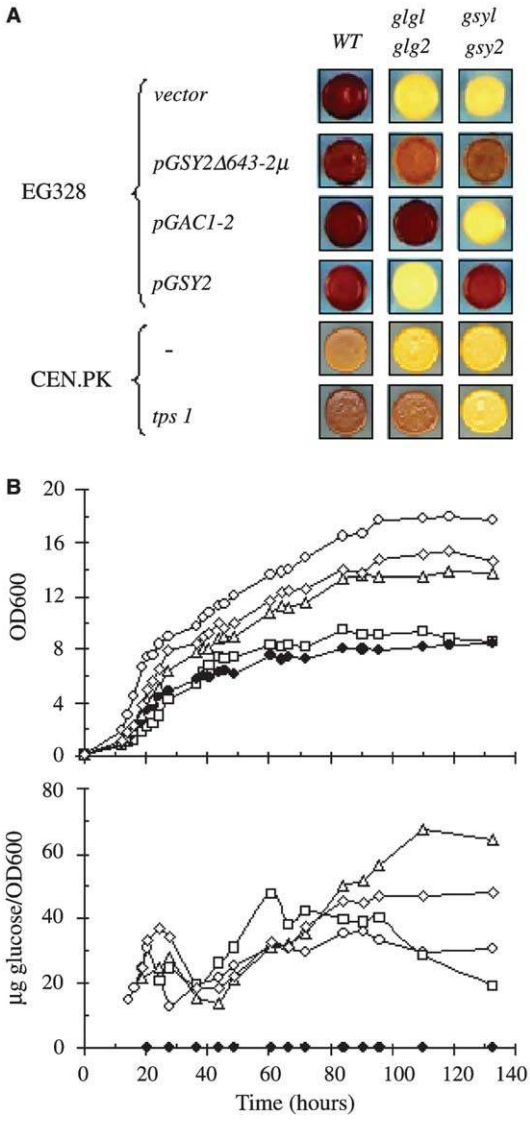

Fig. 2. (A) Iodine staining of cell patches. Strains from the CEN.PK family grown on YNgal: wild-type (CENPK113-7D) and *glg1glg2*, *gsy1gsy2*, *tps1*, *glg1glg2tps1* and *gsy1gsy2tps1* mutant strains. Strains from the EG328 family grown YNglucose: wild-type (EG328-1A) and *glg1glg2* or *gsy1gsy2* derivatives transformed with empty vector, pGSY2, pGSY2Δ 643-2μ or pGAC1-2. (B) Glycogen accumulation during growth of CEN.PK strains in YNGal supplemented with the appropriate auxotrophic requirements: wild-type (○), *glg1glg2* (●), *glg1glg2*+pGAC1-1 (△), *glg1glg2*+pGSY2Δ643-2μ (◇) and *glg1glg2tps1* (□).

Table 1
Metabolites levels in *glg1glg2* and *glg1glg2tps1* mutant cultivated on YNGal medium

	Glycogen synthase (nmol/min/mg protein)*	
	glg1glg2 mutant	*glg2glg2tps1* mutant
− Galactose-6-P	0.80 ± 0.08	1.59 ± 0.04
+ Galactose-6-P 5 mM	2.48 ± 0.12	2.50 ± 0.06
Ratio (−/+ galactose-6-P)	30	62
Metabolites	Intracellular levels (μmol/g dry mass)*	
Glucose-6-P	5.9 ± 0.50	12.5 ± 1.3
Glucose-1-P	5.7 ± 0.65	7.6 ± 1.2
Galactose-1-P	21.5 ± 1.5	41.9 ± 6.0
UDP-glucose	3.85 ± 0.12	4.84 ± 0.15
UDP-galactose	1.25 ± 0.05	1.5 ± 0.15

Enzyme activity and metabolites were measured in cells harvested at the late exponential phase of growth on YNgal ($OD_{600} \approx 3.5$). The values reported are the means ± S.D. of four independent experiments.

cells from this colony were able to regain the capacity to synthesize glycogen. This very low score contrasted with the 2–3% of brown colonies that was obtained when directly spreading a *glg1glg2* culture on plates. When this experiment was repeated with the *glg1glg2tps1* mutant, which restored glycogen synthesis to almost 99% of colonies, more than 90% of the cells that originated from a iodine-positive colony yielded brown colonies, and hence preserved their capacity to accumulate glycogen on successive spreadings (Fig. 3B). Nevertheless, once the glycogen synthesis was lost in a *glg1glg2tps1* colony, the percentage of cells that was able to recover glycogen synthesis was very low (between 0% and 7%). Taken together, these results illustrate a reversible switch between glycogen-positive and -negative phenotypes that does not support a genetic reversion. The data suggest that glycogen synthesis in the absence of glycogenin is a stochastic event that requires a combination of different factors, but is promoted upon deletion of *TPS1*.

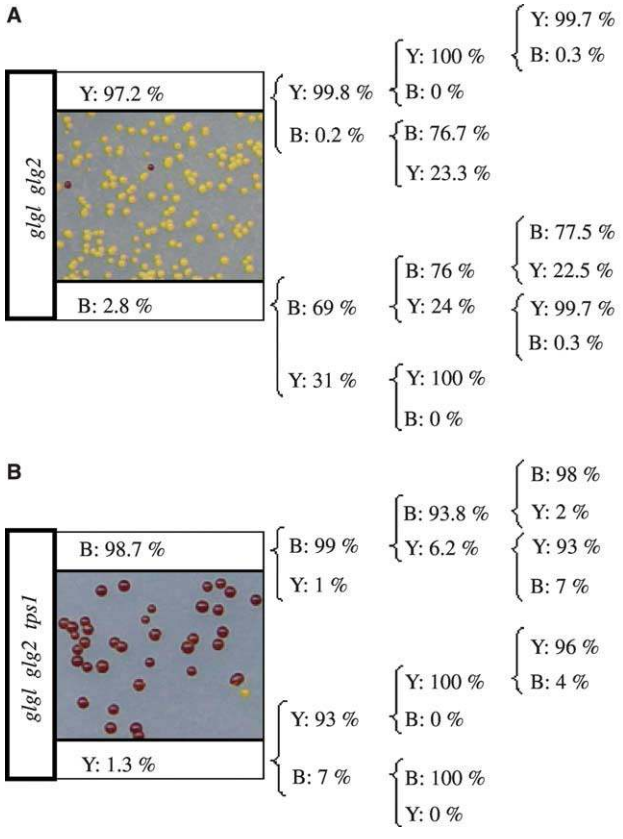

Fig. 3. Stability of the iodine-staining phenotype upon successive passage of colonies from *glg1glg2* (A) and *glg1glg2tps1* (B) mutant strains. The strains were grown on YNGal and spread on nitrogen-limited agar plates to obtain isolated colonies. After two days of growth, they were inverted over iodine vapour, and the percentage of yellow and brown colonies was scored. Single yellow or brown colonies were then independently resuspended in 1 ml sterile water, diluted, and spread to give 100–200 colonies per plate (1 colony $\sim 10^6$ individual cells). Again, the percentage of yellow (Y) or brown colonies (B) was scored. This procedure was repeated two more times.

This stochastic glycogen synthesis was even better illustrated by the heterogeneity in iodine staining within individual colonies of *glg1glg2* cells transformed with pGSY2Δ643-2μ (Fig. 4A). More precisely, these iodine-responsive colonies exhibited yellow sectors, whereas small sectors of brown colour could be seen in yellow colonies. This sectoring pattern is classically observed with phenotypes that are controlled by genes subjected to silencing, as for instance the red colour sectors of colonies when *ADE2* is located at the mating type locus [26]. Also, a fragment of the endogenous 2μ plasmid has been reported to cause gene silencing on adjacent regions [27]. However, this element, which is present in the pGSY2Δ643-2μ construct does not account for *GSY2* silencing and for the switch between the two phenotypes since colour sectors were also observed in colonies of a *glg1glg2* strain expressing a hyperactive Gsy2p from a CEN vector (Fig. 4B). The difference between the two transformants was a higher fraction of brown colonies (16 vs. 26%) that stained more intensely with iodine vapour in the *glg1glg2* mutant transformed by pGSY2Δ643-2μ. In addition, yellow sectors were observed at the periphery of the few iodine-positive *glg1glg2* colonies (Fig. 4C), which support data from Fig. 3A that glycogen synthesis was rapidly lost in this glycogenin defective strain.

4. Discussion

The synthesis of glycogen in yeast cells defective in glycogenin is at first glance unpredictable, based on previous reports showing that deletion of *GLG1* and *GLG2* encoding the two glycogenin abolished this synthesis [4,10]. The ability of a glycogenin-defective strain to accumulate glycogen was nevertheless a rare event found in fewer than 3% of colonies present on agar plates. This capacity was enhanced in the presence of a hyperactive form of glycogen synthase or upon deletion of *TPS1*, which furthermore caused an increase in UDP glucose and glucose-6-P, the substrate and a positive effector of glycogen synthase, respectively. However, it is unlikely that this glycogen synthesis occurs through glycogen synthase alone, since yeast glycogen synthase expressed in *A. tumefaciens* was unable to initiate glycogen synthesis, in contrast to the native bacterial glycogen synthase [7]. Altogether, these results support the existence of an initiator molecule that serves to prime glycogen synthesis. In a previous report, we already speculated on the presence of alternative primers for glycogen initiation, since we found that mutated glycogenin proteins that do not have oligosaccharides attached to them, can still

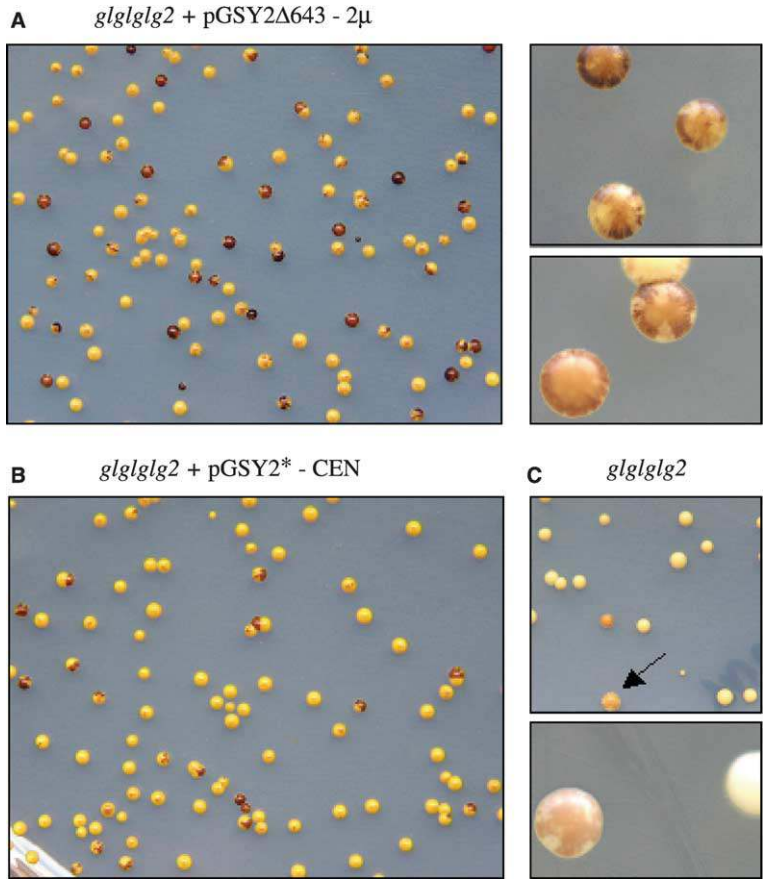

Fig. 4. Colony-sectoring phenotype upon iodine staining. (A) Colonies obtained from a culture of a *glg1glg2* mutant transformed with pGSY2Δ643-2μ. Right panels: magnification of sectored brown colonies showing multiple yellow sectors. (B) *glg1glg2* mutant transformed with pGSY2*-CEN. (C) *glg1glg2* mutant strain. The arrow (upper panel) points to a brown colony with sectoring phenotype (a magnification of one of these colonies is shown in the lower panel).

synthesize ~10% of the wild-type glycogen likely through transglucosylation of alternative primers [10]. The lack of glycogenin in our *glg1glg2* deletion mutant definitively excludes any role for glycogenin in the initial primer glucosylation. The idea that possible acceptor molecules could by-pass the need for glycogenin to prime glycogen synthesis is not without precedent since short oligosaccharides from maltose to maltopentose can be used as artificial acceptors for the transfer of glucose from UDP-glucose by glycogen synthase [28]. However, as the K_m for these acceptors is extremely high (>100 mM), these data likely do not have any physiological relevance. Similarly, alkylglycosides can act as artificial primers for glycogen biosynthesis with a high affinity for glycogen synthase, but their existence in vivo is questionable [8]. Biochemical attempts to identify this potential initiator in yeast cells have so far failed, as glycogen produced from glycogenin mutant could be totally degraded by α-(1,4),α-(1,6) amyloglucosidase (unpublished). This could suggest that the initiator molecule is either an oligosaccharide containing α-(1,4) or α-(1,6) glucosyl linkages or a glucosylated protein that is present at the initiation stage and eliminated later during elongation.

A relevant observation was that all the glycogenin-deficient strains derivatives exhibited clear iodine-staining sectoring within individual colonies. This finding is consistent with this synthesis of glycogen being stochastic in nature, depending on a combination of different factors that are not distributed or transmitted equally between mother and daughter cells. Thus, the 'on–off' glycogen accumulation in cells within a single colony may be accounted for by the presence of a limited amount of the initiator molecule together with a high activity of glycogen synthase. The incidence of these two events would be extremely low in a *glg1glg2* mutant. Alternatively, a key gene involved in the synthesis of this alternative molecule might undergo epigenetic silencing if located near a telomere or in a region subjected to silencing. The finding that the synthesis of glycogen in a *glg1glg2* mutant was enhanced upon deletion of *TPS1* could be consistent with previous data that alteration in trehalose synthesis affects glycogen metabolism [11,29]. However, we could also speculate that the loss of *TPS1* function almost completely releases the epigenetic silencing, if this latter is responsible for the stochastic synthesis of glycogen in the absence of glycogenin. Elucidation of this glycogenin-independent synthesis of glycogen will be challenging due to its stochastic nature but work towards this goal is ongoing.

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