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Marie Ange Teste, Brice Enjalbert, Jean Luc Parrou, Jean M. François *

Centre de Bioingénierie Gilbert Durand, UMR-CNRS 5504, UR-INRA 792, Département de Génie Biochimique et Alimentaire, Complexe Scientifique de Rangueil, 31077 Toulouse Cedex 04, France

Abstract

The *YPR184w* gene encodes a 1536-amino acid protein that is 34–39% identical to the mammal, *Drosophila melanogaster* and *Caenorhabditis elegans* glycogen debranching enzyme. The N-terminal part of the protein possesses the four conserved sequences of the α-amylase superfamily, while the C-terminal part displays 50% similarity with the C-terminal of other eukaryotic glycogen debranching enzymes. Reliable measurement of α-1,4-glucanotransferase and α-1,6-glucosidase activity of the yeast debranching enzyme was determined in strains overexpressing *YPR184w*. The α-1,4-glucanotransferase activity of a partially purified preparation of debranching enzyme preferentially transferred maltosyl units than maltotriosyl. Deletion of *YPR184w* prevents glycogen degradation, whereas overexpression had no effect on the rate of glycogen breakdown. In response to stress and growth conditions, the transcriptional control of *YPR184w* gene, renamed *GDB1* (for Glycogen DeBranching gene), is strictly identical to that of other genes involved in glycogen metabolism.

Keywords: Glycogen; Debranching enzyme; Functional analysis; Stress; *Saccharomyces cerevisiae*

1. Introduction

Glycogen is a large branched polymer of glucose residues, which accumulates in yeast in response to nutrient limitation and to stress such as heat, osmotic and saline stress, and to nutrient starvation [1–3]. Resumption of growth of non-proliferating yeast cells or addition of uncouplers to resting cells is accompanied by a rapid mobilisation of glycogen [4]. This degradation occurs by the combined action of two enzymes: the glycogen phosphorylase and the glycogen debranching enzyme. The former (EC 2.4.1.1) catalyses the sequential phosphorolysis of α-1,4-linked glucose units until two or three glucose units remain before a α-1,6-branch point [5,6]. The second enzyme eliminates the branch point in a two-step process that includes (i) the transfer of a maltotriosyl (or maltosyl) unit from the branch to an adjacent α-1,4-glucosyl chain by its oligo-1,4→1,4-glucanotransferase activity (EC 2.4.1.25), and (ii) the subsequent hydrolysis of the residual α-1,6-linked glucose residue by its α-1,6-glucosidase activity (EC 3.2.1.33). A direct debranching enzyme termed isoamylase which can attack glycogen or amylopectin by hydrolysing α-1,6-linkages exists in bacteria but its occurrence in yeast is still controversial [7]. Debranching enzymes have been purified from several organisms including *Escherichia coli* [8], yeast [5,7] and mammals [9–11]. They all bear transferase and glucosidase activities on a single polypeptide with a molecular mass of 160–170 kDa. Various systems have been developed based on substrate specificity for measurement of the glucanotransferase, α-glucosidase or both activities of the debranching enzyme [12]. Evidence that the two reactions take place at two distinct catalytic sites was provided by enzymological studies, using inhibitors specifically affecting either the transferase or the glucosidase activity [13]. Same conclusions were given from a partial proteolysis of the purified enzyme with trypsin or chemical reactivity with carbodiimide, which resulted in the loss of the transferase activity without affecting α-glucosidase activity [10,14]. Furthermore, the presence of the four conserved consensus sequences of the α-amylase superfamily in the N-terminal sequence of the mammalian debranching enzyme [15] led the authors to suggest that the transferase activity may encompass the N-terminal part of the protein, leaving the α-glucosidase

* Corresponding author. Tel.: +33 (5) 61559492; Fax: +33 (5) 61559400; E-mail: fran_jm@insa-tlse.fr

activity for the C-terminal part [16]. There is still no physical demonstration that the two activities of the debranching enzyme are functionally present in two distinct domains.

In yeast, most of the structural genes encoding enzymes involved in the biosynthesis and biodegradation of glycogen have been cloned and characterised (for a review, see [17]), except the gene encoding the debranching enzyme. The *YPR184w* gene sequence on the yeast chromosome 16 encodes a protein 39% identical to mammals glycogen debranching enzyme. In human, the deficiency in liver and muscle glycogen debranching enzyme causes the glycogen storage disease type III which results in a hyperaccumulation of branched glycogen [18]. In this report, we showed that the *YPR184w* open reading frame (ORF) encodes the yeast Glycogen DeBranching enzyme and that this gene, renamed *GDB1*, is essential for glycogen degradation in yeast.

2. Materials and methods

2.1. Yeast strains, media and culture conditions

Strains KT1112 (*Mata his3Δ200 leu2-3, 112 ura3-52*), JF624 (*Matα leu2-3, 112 ura3-52 his3 trp1Δ1 lys2-801*) and its isogenic diploid strain JF626 were used as hosts for transformations. The haploid strain JF784 (*Matα leu2-3, 112 ura3-52 his3 trp1Δ1 lys2-801 gph1::LEU2*) is deleted for *GPH1* encoding the yeast glycogen phosphorylase. A 3800-bp *Xho*I–*Xho*I fragment removed from YCpX:(*gph1Δ:LEU2*) [19] was used to disrupt *GPH1*. Yeast cells were grown at 30°C on YEP medium (1% yeast extract plus 2% bactopeptone) supplemented with 1% instead of 2% glucose, or on yeast nitrogen base (YNB) medium (0.17% of YNB without amino acids and ammonium sulphate plus 0.5% ammonium sulphate and 1% glucose) supplemented with 100 μg ml^{-1} of the auxotrophic requirement [20]. The YNB medium also contained 1% (w/v) succinic acid and 0.6% (w/v) NaOH to keep the pH around 5.8. To promote glycogen mobilisation, two types of experiments were carried out. A first experimental procedure was to dilute by 10 times a culture of late exponential yeast cells grown on YNB (optical density at 600 nm (OD_{600}) ≈ 2.0) into a fresh YNB medium and to collect 20-ml samples at different times for glycogen assays. The second experiment took the advantage that the addition of uncouplers to yeast cells stimulates glycogen degradation [4]. To this end, cultures of late exponential yeast cells were harvested by centrifugation and washed once with water. The cells were resuspended at $OD_{600} = 20$ into a nitrogen-free medium (50 mM Na-succinate, 5 mM KCl, 12 mM NaPi, pH 5.5) in the presence of glucose 2% (w/v). After 90 min of incubation at 30°C, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) from a 20-mM stock solution made in methanol was added to the cells (0.2 mM final concentration), and 2-ml samples were collected by centrifugation (2 min at 3000 × *g*) at different times for glycogen assay.

2.2. Plasmids and strains construction

The *YPR184w* or *GDB1* gene was cloned as a 8440-bp *Stu*I–*Kpn*I fragment derived from cosmid 9705 (purchased from ATCC no. 71011) into pBSK to yield pMA2. A 3717-bp *Bgl*II–*Bgl*II which removed 80% of the *GDB1* coding sequence was replaced by a 1.1-kbp *Bam*HI (*TRP1*) fragment from YDpw [21] to yield pMA3. The 2640-bp *Nar*I–*Kpn*I fragment from this plasmid was integrated into the chromosomal locus of *GDB1* in the diploid strain JF626 by classical yeast transformation procedure [22]. The haploid strain JF937 bearing the *gdb1::TRP1* allele was obtained after sporulation and tetrads dissection. Construction of *GDB1-lacZ* fusion gene was made by PCR amplification of a 1014-bp fragment from the *GDB1* promoter with the two following primers: GDB1-up 5′-CGCGGATCCCGCAGACCTACAACA and GDB1-dw 5′-ATGCCTGCAGGCAGTAATGATCTATT (the *Bam*HI and *Pst*I sites are underlined). The *Bam*HI–*Pst*I PCR fragment was inserted into the *Bam*HI–*Pst*I site of YIp356 [23] to yield pMA5. Recombinant PCR protocol [24] was applied in order to replace the unique STRE of the *GDB1* promoter by a *Nhe*I site using four oligomers: GDB1-up and GDB1-dw as external primers, and Nhe15 (GCAAGCTAGCGAAATGGCCAATTCTTCTCG) and Nhe13 (TTGGCCATTTCGCTAGCTTGCTGCTGTTGTGACAAAAAAG) as internal primers (underlined is the *Nhe*I site which replaces the STRE). Insertion of the PCR fragment in YIp356 yielded YIpΔSTRE-*gdb1*. pMA5 and YIpΔSTRE-*gdb1* were digested with *Nco*I and integrated at the *ura3-52* locus in strain KT1112 by homologous recombination [22] to yield JF1200 and JF1201. Transformants were verified by Southern blot analysis (data not shown). For experiments of glycogen debranching enzyme overproduction, the PCR-amplified *GDB1* coding sequence was cloned in frame to *PGK1* promoter in pYGE2 [25]. The ORF was amplified using GDB-surex5 (5′-CGCTGACCCGGGATGAAATAGATCATTACTGCTACGT) and GDB-surex3 (5′-GGCGCCGGTACCTCAGGAATCATCTTCGTAGGCATC) (the restriction sites *Sma*I and *Kpn*I are underlined). The amplified fragment was cloned into pBSK and a 4.4-kbp *Kpn*I–*Kpn*I fragment was recloned into the unique *Kpn*I site of pYGE2 to yield pMA9.

2.3. Biochemical and analytical procedures

The glycogen debranching enzyme was assayed by four different methods. A quick assay is based on the increase in the iodine staining intensity with limit dextrin [26]. Samples (210 μl) were incubated at 30°C with 0.1% (w/v) of glycogen phosphorylase limit dextrin prepared according

to Lee et al. [5] in 50 mM citrate sodium pH 6.0 in a total volume of 0.3 ml. After 1 h of incubation, the reaction was mixed with 5 µl of an iodine staining solution (0.2% I_2–2% KI) and 5 µl of HCl 2 M, and the OD was read at 550 nm. The combined activity of the glucanotransferase and α-1,6-glucosidase was measured by the release of glucose from glycogen phosphorylase limit dextrin as described in [5]. The glucanotransferase activity of the debranching enzyme was determined at 30°C in 50 mM Na-citrate pH 6.0 in the presence of 50 mM maltopentaose as the substrate. The different oligosaccharides generated by this activity were separated and quantified by high performance anionic exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, Sunnyvale, CA, USA) according to [27]. Finally, the amylo-1,6-glucosidase activity was estimated by the incorporation of [U-^{14}C]-glucose into glycogen following the procedure of Hers et al. [28], with the exception that the buffer was Na-citrate 50 mM, pH 6.0 and the final concentration of [U-^{14}C]glucose in the assay was 25 mM (specific activity of 2000 dpm nmol^{-1}).

For partial purification of the debranching enzyme, 1 g of yeast cells (wet weight) grown to stationary phase on YNB was resuspended in 4 ml of ice-cold solution containing 50 mM Tris–Cl, pH 7.5, 1 mM EDTA, 1 mM phenylmethyl sulphonate fluoride and 10 µg ml^{-1} of a protease cocktail inhibitors (complete EDTA free protease inhibitors from Roche Diagnostic). The cells were disrupted with 4 g glass beads (0.4–0.5 mm diameter) by vigorous shaking on a vortex for five 30-s periods at 1-min intervals. The extract was centrifuged for 5 min at $3000 \times g$. The nucleic acids were precipitated by addition to the supernatant of protamine sulphate (purchased from Sigma) at a final concentration of 0.1% (w/v). After 10 min of incubation on ice, the crude extract was centrifuged for 30 min at $20\,000 \times g$. The supernatant was filtered through a minisart filter (0.2 µm porosity) and applied onto a Resource 15 Q column (FPLC system from Pharmacia Biotech) equilibrated with 50 mM Tris–Cl, pH 7.5. After washing the column with 5 ml of Tris–Cl solution, a continuous gradient of NaCl (0–500 mM) made in this buffer solution was applied in a FPLC system. Fractions of 1 ml were collected and the iodine staining method was used to identify the presence of debranching enzyme activity. Protein concentrations were determined by the method of Bradford [29] using bovine serum albumin as a standard. Cell samples and assays of glycogen and β-galactosidase were performed as described [2].

3. Results and discussion

3.1. Sequences comparison with debranching enzymes from various organisms

In *Saccharomyces cerevisiae* databases (see for instance the YPD database), the *YPR184w* is assigned as an ORF encoding a putative glycogen debranching enzyme, because it encodes an 174 845 kDa protein that exhibits between 34 and 39% identity with *Drosophila melanogaster*, *Caenorhabditis elegans* and mammals (*Homo sapiens* and rabbit) debranching enzymes. This identity is distributed all over the 1536-amino acid protein sequence but two distinct regions with interesting features may be distinguished in the sequence. The N-terminal half of the protein (750 amino acids) possesses the four consensus sequences commonly found in the α/β barrel domain of α-amylase and α-glucanotransferase [15], suggesting that this region may encompass the oligo-1,4 → 1,4-glucanotransferase activity of the debranching enzyme. The 800 residues at the C-terminus may correspond to a second functional domain, since it shows 50% similarity with the C-terminal part of the debranching enzymes from other eukaryotic species. According to Liu et al. [16], this domain could bear the amylo-1,6-glucosidase activity of the debranching enzyme. However, all enzyme hydrolysing α-1,6-glucosidic linkages also display the four regions that are conserved among the α-amylase superfamily [15], suggesting that the N-terminal of the protein is required for the glucosidase activity. The only pertinent difference at the protein sequence level between specific α-1,4-amylases and amylo-1,6-glucosidases is at the level of the consensus II (Fig. 1) in which two amino acids (K and H) are not conserved in these latter enzymes [30]. A potential function of the C-

	I	II	III	IV
AMYpp	90-GVRIYV**D**AVI**NH**-101	192-AGF**R**L**D**ASKH-201	229-FIFQ**E**VID-236	292-ALVFVDN**HD**-300
CGTbm	129-NIKVVM**D**FAP**NH**-140	224-DGI**R**F**D**AVKH-233	253-FTFG**E**WFL-260	320-MVTFIDN**HD**-328
ISAps	285-GIKVYM**D**VVY**NH**-296	369-DGF**R**F**D**LASV-378	412-RILR**E**FTV-419	499-SINFIDV**HD**-507
GDEhs	229-NVICIT**D**VVY**NH**-240	521-QGV**R**L**D**NCHS-530	551-YVVA**E**LFT-558	619-ALFMDIT**HD**-627
GDErb	232-NVLCIT**D**VVY**NH**-243	544-QGV**R**L**D**NCHS-553	574-YVVA**E**LFT-581	642-ALFMDIT**HD**-650
GDBce	196-NILTVQ**D**VVW**NH**-207	512-HGL**R**I**D**NAHG-521	542-YVFA**E**LFT-549	610-GLFLDQS**HD**-618
GDBdm	229-GVASIC**D**IVL**NH**-240	551-DGV**R**L**D**NCHS-560	581-YVVA**E**LFT-588	649-ALFLDLT**HD**-657
GDBsc	218-NMLSLT**D**IVF**NH**-229	530-DGF**R**I**D**NCHS-539	560-YVVA**E**LFS-567	662-ALFMDCT**HD**-670

Fig. 1. Predicted amino acid sequence alignment of the yeast with other debranching enzymes. Conserved sequence in the α-amylase superfamily. Enzymes are numbered from the N-terminal end. Invariable residues are in boldface. AMYpp (α-amylase pig pancreas), CGTbm (cyclodextrin glycosyltransferase of *Bacillus macerans*), ISAps (isoamylase from *Pseudomonas amyloderamosa*), GDEhs (glycogen debranching enzyme from human liver), GDErb (glycogen debranching enzyme from rabbit), GDBce (glycogen debranching enzyme from *C. elegans*), GDBdm (glycogen debranching enzyme from *D. melanogaster*) and GDBsc (glycogen debranching enzyme from *S. cerevisiae*).

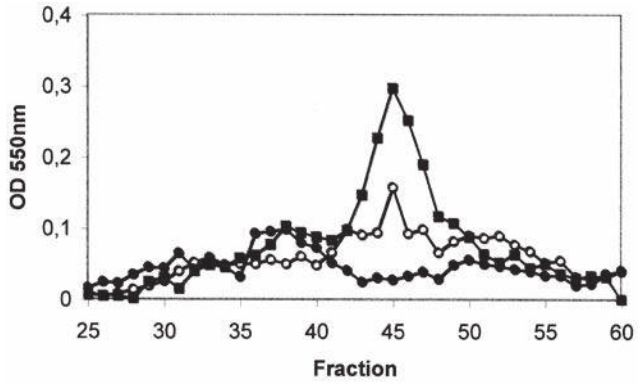

Fig. 2. Elution profile of debranching enzyme from a Resource 15 Q column chromatography. Proteins (about 15 mg) prepared from wild-type (○), *gdb1* mutant (●) or *GDB1* overexpressing mutant (■) were loaded on a Resource 15 Q column. Activity of glycogen debranching was determined by iodine staining method as described in Section 2.

terminal domain of the debranching enzyme could be in the binding of glycogen as suggested by Chen and colleagues [31]. This suggestion was based on a short sequence identity between two segments at the C-terminal region of the human muscle debranching enzyme and the glycogen-binding region of glycogen phosphorylase. However, these segments were not found in the yeast protein. Preliminary experiments showed the deletion of a 555-bp *Sph*I fragment in the C-terminal part of the *YPR184w* gene abolished both activities, indicating that this C-terminus is required for both activities of the debranching enzyme. Finally, it is interesting to point out that *S. cerevisiae*, *C. elegans* and mammals debranching enzyme bear little similarity to *E. coli* and starch R-debranching enzyme [32,33], possibly because the structure of the glucose polymers is less ramified in *E. coli* and in starch than that in yeast and mammals.

3.2. Determination of glycogen debranching activity in wild-type and GDB1 overexpressing strains

The debranching enzyme can be assayed by several methods, which illustrates α-glucosidase [27], transferase [6] or both activities [5]. Since wild-type cells con tained very low debranching activity (about 0.4 nmol [U-^{14}C]-glucose incorporated in glycogen per hour and per mg proteins for the amylo-1,6-glucosidase assay), we developed a partial purification of this enzyme by loading 15 mg proteins from crude extracts on an anionic exchange column followed by elution with a NaCl gradient. The assay by the iodine staining method identified debranching activity in peak fractions eluting at ca. 280 mM NaCl (fractions 43–47). These fractions contained about four times more activity when the same amount of protein of a crude extract from a strain overexpressing *GDB1* was loaded on the column, whereas they were absent in the extract from a *gdb1* null mutant (Fig. 2). The iodine assay was confirmed by quantitative measurement of glucose released from glycogen phosphorylase dextrin limit (not shown). Moreover, α-1,6-glucosidase and transferase activities were determined on fraction no. 45 from the overproducer strain. The α-glucosidase activity incorporated 10.5 nmol [U-^{14}C]glucosyl units in glycogen per min per ml of fraction. The incubation with 50 mM maltopentaose for 1 h at 30°C resulted in a higher production of maltotriose (74 nmol ml^{-1} of fraction) and maltoheptaose (63 nmol ml^{-1}) than maltose (25 nmol ml^{-1}) and maltooc-

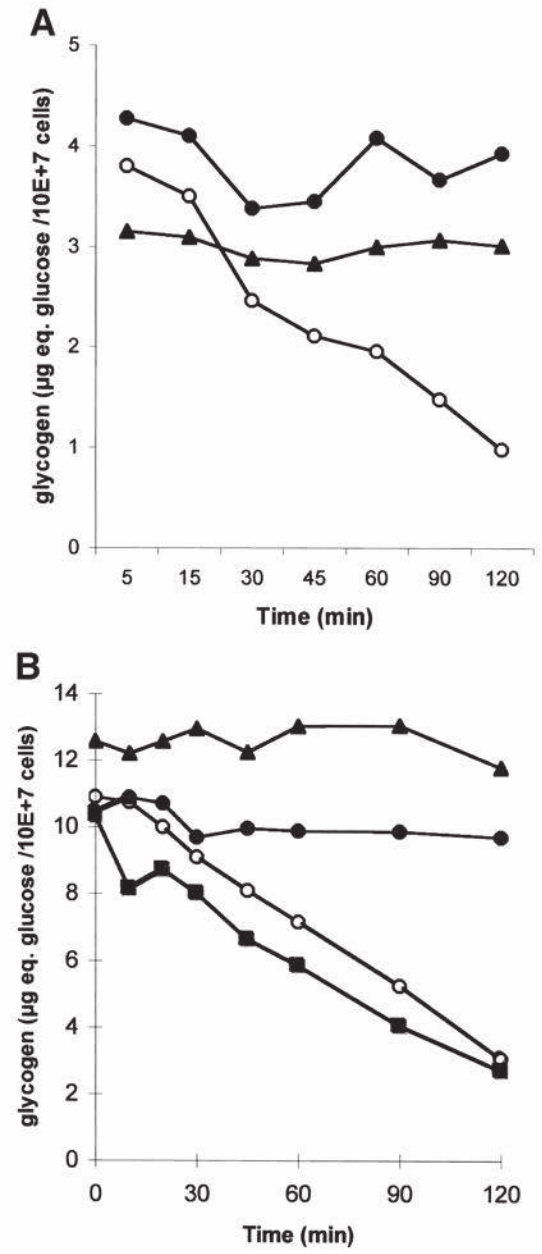

Fig. 3. Effect of loss of *GDB1* function on glycogen degradation upon resuspension of stationary phase yeast in fresh growth medium (A) or in response to addition of CCCP to suspension of yeast (B). Strains used in this experiment were JF624 (WT; ○), JF937 (*gdb1Δ::TRP1*; ●); JF784 (*gph1Δ::LEU2*; ▲) and JF624 transformed with pMA9 (*GDB1* under the *PGK1* promoter, ■). CCCP was added at a final concentration of 0.2 mM.

taose (25 nmol ml^{-1}), which confirmed that the yeast debranching enzyme preferentially transfers two glucose units on an adjacent linear chain [5,6].

3.3. Physiological effect of deletion and overexpression of *GDB1*

As shown in Fig. 3A, the transfer of glycogen-containing cells from stationary phase to a fresh growth medium triggered the degradation of the polysaccharide. As expected, this event was completely abolished in a *gph1* mutant, and interestingly, it was also prevented in a *gdb1* null mutant. Reintroduction of the wild-type *GDB1* in the mutant strain restored normal glycogen degradation (not shown). Glycogen mobilisation can also be triggered by the addition of uncouplers, such as CCCP to yeast cells incubated in a nitrogen-free glucose medium [4]. This CCCP-induced glycogen mobilisation was abolished upon deletion of *GPH1* or *GDB1* (Fig. 3B). Overexpression of *GDB1* gene under the control of a *PGK1* promoter had no consequence on the rate of glycogen degradation (Fig. 3B), supporting the notion that the rate-limiting step in this process is determined by glycogen phosphorylase [17]. Taken together, these results demonstrate that the in vivo degradation of glycogen requires the presence of both glycogen phosphorylase and glycogen debranching enzyme. It is possible that the inability of glycogen phosphorylase alone to reduce the glycogen content is due to the fact that the α-1,6-branched points of the glycogen molecule restrict the action of glycogen phosphorylase at the polymer periphery.

3.4. Expression of GDB1 during the diauxic growth on glucose and in response to various stress

Previous works demonstrated a coordinate regulation at the level of transcription of genes involved in glycogen biosynthesis and biodegradation under various nutritional and stress conditions ([3], and reference therein). Using an integrated *GDB1-lacZ* fusion gene, it is shown that the increase in β-galactosidase activity from this construct during diauxic growth on glucose strictly followed the transcriptional patterns found with the other genes involved in reserve carbohydrates metabolism (Fig. 4 and [3]). As in the case of *GSY2* [34], the removal of the single STRE element located at −191 bp upstream of the ATG codon of the *GDB1* ORF did not prevent its growth-related transcriptional induction although reducing by 4-fold the β-galactosidase activity from *GDB1-lacZ* construct. In contrast, the 3-fold increase of *GDB1-lacZ* expression in response to a temperature shift from 25 to 37°C and its 2-fold increase in response to the addition of 0.3 M NaCl were abolished upon elimination of the STRE element (results not shown). These results strengthen the absolute necessity of STRE element for gene activation in response to stress conditions, while the coordinated – STRE independent – transcriptional induction during the diauxic growth on glucose [34,35] is indicative of a singular, not yet identified, sensing mechanism coordinating growth and cell division.

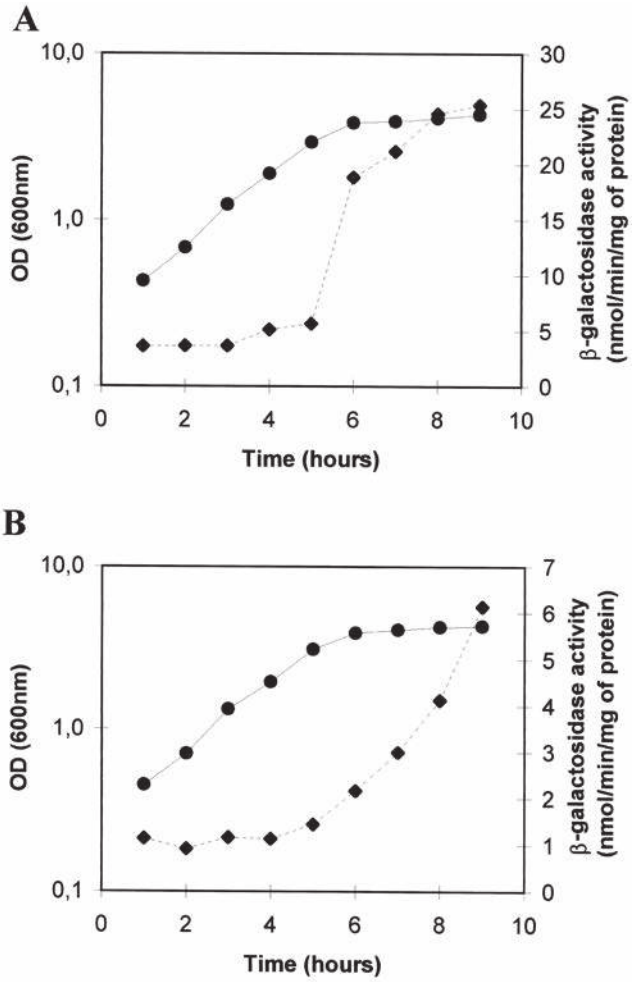

Fig. 4. Expression patterns of *GDB1-lacZ* and ΔSTRE-*gdb1-lacZ* translational fusion during the diauxic growth on glucose. Strains JF1200 (*GDB1-lacZ* gene fusion, A) and JF1201 (ΔSTRE-*gdb1-lacZ* construct, B) were grown on YEP in the presence of 1% glucose. Symbols are: cell growth (●), β-galactosidase (♦).

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