RESEARCH LETTER

Metabolism of D-galactose is dispensable for the induction of the *beta*-galactosidase (*bgaD*) and lactose permease (*lacpA*) genes in *Aspergillus nidulans*

Anita Orosz, Erzsébet Fekete, Michel Flipphi & Levente Karaffa

Department of Biochemical Engineering, Faculty of Science and Technology, University of Debrecen, Debrecen, Hungary

Correspondence: Levente Karaffa, Department of Biochemical Engineering, Faculty of Science and Technology, University of Debrecen, H-4032 Debrecen, Hungary. Tel.: +36 52 512 900 ext. 62488; fax: +36 52 533 677; e-mail: karaffa.levente@science.unideb.hu

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Abstract

In this study, we analyze the expression of the *Aspergillus nidulans bgaD-lacpA* gene couple (encoding an intracellular beta-galactosidase and a lactose permease) in the presence of D-galactose. This monosaccharide can be catabolized via alternative, independent pathways in this model organism. The inductive capabilities of intermediates of the two alternative routes of D-galactose utilization were addressed in loss-of-function mutants defective in a defined step in one of the two pathways. In a galactokinase (*galE9*) mutant, the cluster is strongly induced by D-galactose, suggesting that formation of Leloir pathway intermediates is not required. The expression profiles of *bgaD* and *lacpA* were similar in wild type, L-arabinitol dehydrogenase (*araA1*), and hexokinase (*hxkA1*) negative backgrounds, indicating that intermediates of the oxido-reductive pathway downstream of galactitol are not necessary either. Furthermore, *bgaD-lacpA* transcription was not induced in any of the tested strains when galactitol was provided as the growth substrate. An *hxkA1/galE9* double mutant cannot grow on D-galactose at all, but still produced *bgaD* and *lacpA* transcripts upon transfer to D-galactose. We therefore concluded that the physiological inducer of the *bgaD-lacpA* gene cluster upon growth on D-galactose is the nonmetabolized sugar itself.

Introduction

The heterodisaccharide lactose (1,4-O-β-D-galactopyranosyl-D-glucose) occurs mainly in mammalian milk where it makes up 2–8% of the dry weight, and it is an abundant byproduct of the dairy industry (Roelfsema *et al.*, 2010). Technical-scale hydrolysis of lactose – a challenging process in food and fermentation industry – is catalyzed by microbial (typically fungal) β-D-galactosidases (bGal). Fungal bGals can be distinguished into glycosyl hydrolase family (GH) 35, which contains extracellular enzymes characterized by an acidic pH optimum, and GH2 which comprises intracellular ones, that function optimally at neutral pH (Fekete *et al.*, 2002, 2012; Ishikawa *et al.*, 2005; Gamauf *et al.*, 2007). Purification and biochemical characterization of bGal enzymes have been described for a wide range of fungal sources (for a review, see Panesar *et al.*, 2006), but regulation of expression of the encoding genes is still not well understood.

Hydrolysis of lactose yields D-glucose and D-galactose. In ascomycete filamentous fungi, D-galactose catabolism can proceed via two pathways, that is, the canonical Leloir pathway that begins with an ATP-dependent phosphorylation of the pyranoside hemiacetal (circular) form that yields D-galactose-1-phosphate and eventually results in D-glucose-1-phosphate (Roberts, 1970; Alam & Kaminskyj, 2013), and the oxido-reductive pathway that resembles the one described for L-arabinose, and where D-galactose in the open (linear) configuration is reduced first into galactitol (De Vries *et al.*, 1994; Fekete *et al.*, 2004; Seiboth *et al.*, 2007). This pathway would eventually yield fructose-6-phosphate or tagatose-1,6-bisphosphate in *Aspergillus nidulans* (Fig. 1).

Aspergillus nidulans is a model system for biochemical and genetic research in multicellular fungi (for a recent review, see Yaegashi *et al.*, 2014). In this fungus, a lactose and D-galactose inducible intracellular β-galactosidase activity with a neutral pH optimum has been described whose expression also modestly responds to the presence of L-arabinose (Fekete *et al.*, 2002, 2012). In analogy with the well-studied *Kluyveromyces lactis* lactose assimilation system (e.g. Gödecke *et al.*, 1991; Diniz *et al.*, 2012), we

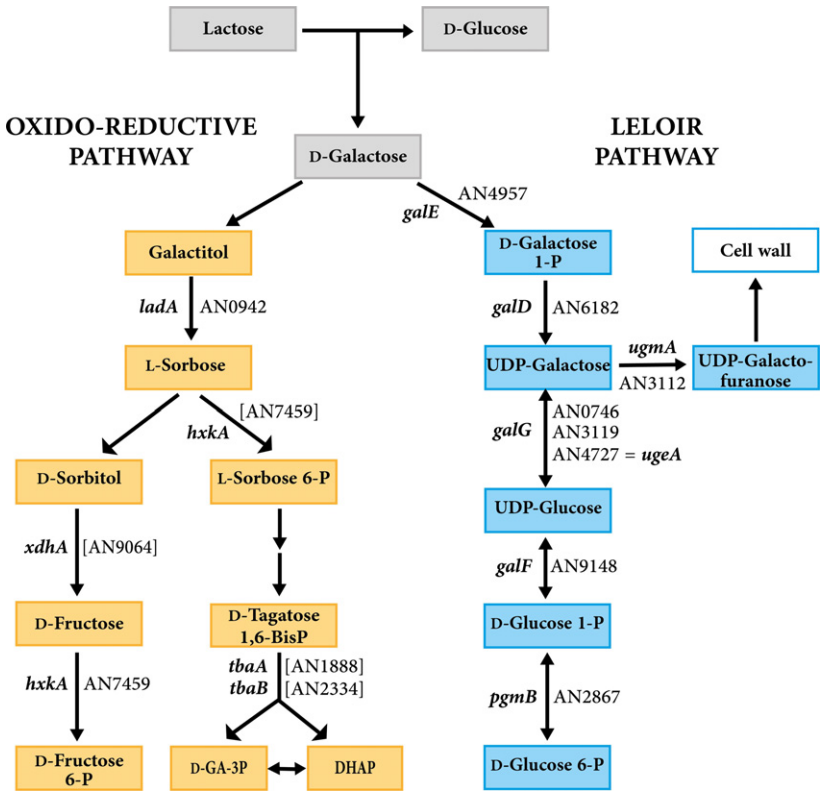

Fig. 1. D-galactose catabolism in *Aspergillus nidulans*. The (*a priori* nonmutually exclusive) alternatives for the oxido-reductive route are depicted as proposed by Fekete *et al.* (2004) and Flipphi *et al.* (2009) and both involve L-sorbose as the product of galactitol oxidation (step 2). The *A. nidulans* loci putatively involved are given between square brackets. The anabolic functions of the Leloir route imply that most Leloir enzymes are expressed constitutively during vegetative growth. The UDP-glucose/galactose 4-epimerase (*ugeA*) and UDP-galactopyranose/furanose mutase (*ugmA*) genes and their role in cell wall synthesis were described by El-Ganiny *et al.* (2008, 2010). Hexokinase mutant *hxkA* cannot grow on D-fructose but grows normally on D-glucose by virtue of the fungus' glucokinase activity (Flipphi *et al.*, 2003). D-GA-3P: D-glyceraldehyde-3-phosphate; DHAP: dihydroxy-acetone-phosphate.

recently identified and characterized two clustered, divergently transcribed genes encoding an intracellular β-galactosidase (*bgaD*) and a lactose permease (*lacpA*; Fekete *et al.*, 2012). These two genes were expressed to basal levels in carbon-derepressed (creA^{d}) mutant backgrounds, even when D-glucose was the only growth substrate present. By creating deletion mutants, we provided evidence that *bgaD* is the only enzyme in *A. nidulans* that acts on the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) – generally regarded as a typical albeit artificial β-galactosidase substrate – while *lacpA* was demonstrated to mediate high affinity uptake of lactose. Here, we show that the induction of the catabolic *bgaD-lacpA* gene couple observed on D-galactose is strictly mediated by the monosaccharide rather than by an intermediate of its catabolism.

Materials and methods

Aspergillus nidulans strains, media, and culture conditions

Aspergillus nidulans strains used in this study are listed in Table 1. The modified aspergillus minimal medium (AMM2) for shake flask cultures and for submerged bioreactor cultivations (henceforth referred to as 'fermentations') was formulated and inoculated as described by Fekete *et al.* (2002). AMM2 is similar to AMM (Pontecorvo *et al.*, 1953) but including ammonium dihydrogen phosphate ($NH4H2PO4$; 8 g L^{-1}) as the sole nitrogen source, as well as 0.1 g L^{-1} calcium dichloride ($CaCl_2$). Vitamins and other supplements were added from sterile stock solutions. Carbon sources were used at 1% (w/

Table 1. *Aspergillus nidulans* strains used in this work

Strain	Genotype	Property	References
Wild-type (R21)	*pabaA1, yA2*	–	Fantes & Roberts (1973)
A214	*galE9, biA1, wA3*	Galactose kinase negative	Roberts (1963, 1970) Alam & Kaminskyj (2013)
A59 (G092)	*hxkA1, yA2, pyroA4*	Hexokinase negative	Roberts (1963) Flipphi *et al.* (2003)
G094	*biA1, wA3, araA1*	L-Arabinitol dehydrogenase negative	Clutterbuck (1981) De Vries *et al.* (1994)
EFES3	*yA2, galE9, hxkA1, pyroA4*	Hexokinase and galactose kinase negative	Fekete *et al.* (2004)

v) initial concentration unless stated differently. Cultures were inoculated with 5×10^6 *A. nidulans* conidia per millilitre of medium. Shake flask cultures were incubated at 37 °C in 500-mL Erlenmeyer flasks containing 100 mL of culture medium in a rotary shaker at 200 rotations per minute (r.p.m.).

For induction experiments, replacement cultures were used for which mycelia were pregrown for 24 h in AMM2 medium containing glycerol as the carbon source, and harvested by filtration over a sintered glass funnel. After a thorough wash with cold sterile water, biomass was transferred to flasks with fresh AMM2 containing the various carbon sources tested. For transcript analysis, samples were taken 3, 6, and 19 h after the transfer of mycelia (also indicated in the respective figures), indicating an early, middle, and late stage of the induction process.

Fermentations were inoculated with harvested and washed pregrown biomass of 200 mL AMM2/glycerol cultures, and were carried out in a 2.5-L glass bioreactor (Sartorius, Göttingen, Germany) with a culture (working-) volume of 2 L, and equipped with one-six-blade Rushton disc turbine impeller. Operating conditions were pH 6.8, 37 °C, and 0.5 vvm (volumes of air per volume of liquid per minute). The dissolved oxygen level was maintained at 20% saturation and was controlled by means of the agitation rate. To minimize medium loss, the waste gas was cooled in a reflux condenser connected to an external cooling bath (4 °C) before exiting the system.

Preparation of cell-free extracts

Cell-free extracts were prepared as described in Fekete *et al.* (2002). Briefly, suction-filtered biomass was washed with 0.1 M sodium phosphate buffer, pH 6.8. The mycelia were resuspended in the same buffer, then homogenized and centrifuged. The supernatant was immediately used to assay intracellular β-galactosidase activity.

Enzyme assay

β-Galactosidase activity was assayed by incubating 0.5 mL of crude mycelial extract with 0.3 mL of 10 mM *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) and 0.2 mL of distilled water for 30 min at 37 °C. Thereafter, reactions were terminated by the addition of 2 mL 1 M di-sodium carbonate (Na_2CO_3), and the absorbance read at 410 nm. Blanks, in which Na_2CO_3 had been added prior to the addition of ONPG, were always included and considered in the calculations. One unit of β-galactosidase activity corresponds to the release of 1 μmol *ortho*-nitrophenol per minute under the conditions of the assay. Specific activities are reported per mg of protein, the latter being determined using a modification of the method of Lowry (Peterson, 1983) utilizing BSA for calibration.

Nucleic acid isolation and hybridization

Samples of *A. nidulans* mycelia were collected and processed as described by Fekete *et al.* (2012). For nucleic acid isolation, frozen biomass was ground to dry powder under liquid nitrogen, using chilled mortar and pestle. Genomic DNA and total RNA were extracted using Macherey-Nagel's NucleoSpin Plant II DNA and NucleoSpin RNA Plant isolation kit, respectively, according to the manufacturer's protocol. Standard methods were used for electrophoresis, blotting, and hybridization of nucleic acids. To generate digoxigenin-labeled gene-specific probe material for Northern blot analysis, a PCR DIG probe synthesis kit (Roche) was used following the manufacturer's instructions. The sequences of the oligonucleotides utilized to generate the hybridization probes used are listed in Table 2.

Analytical methods

Mycelial dry weight (DCW) was determined from 20 mL culture aliquots. The biomass was harvested and washed on a preweighted glass wool filter by suction filtration and the filter dried at 80 °C until constant weight. The concentration of galactitol in growth media was determined by HPLC (Fekete *et al.*, 2004).

Reproducibility

All the data presented are means of 3–5 independent experiments (= biological replicates). The data were analyzed and visualized by SIGMAPLOT (Jandel Scientific, San Jose, CA), and standard deviations (SDs) were determined for each procedure. The SD values were always < 14% of the mean values.

Table 2. Oligonucleotides used in this work. The *bgaD* and *lacpA* genes were previously characterized (Fekete *et al.*, 2012)

Gene abbreviation	Activity function	Locus ID	Oligonucleotide sequence (5′–3′)	Amplicon size (bp)
bgaD	Intracellular beta-galactosidase	AN3201	bgaDfw: 5′-AGACCTCGCTGCTGCTGAAC bgaDrv: 5′-GGTCGGGTCTTCTTCCTTTATG	1461
lacpA	Lactose permease	AN3199	lacpAfw: 5′-TTTTTGAATTCGCTGGCTGATGG lacpArv: 5′-TTTTTGAATTCGTGCGGTCTTTGG	796

Chemicals

Except where explicitly noted, all chemicals used were of analytical grade and purchased from Sigma-Aldrich Kft., Budapest, Hungary.

Results

The Leloir pathway is dispensable for *bgaD/lacpA* expression upon D-galactose induction

As may be expected for clustered genes, *bgaD* and *lacpA* displayed essentially identical expression patterns under all physiological conditions tested, independently of the genotype of the galactose-utilization mutants. To identify the physiological inducer of *bgaD/lacpA* upon D-galactose induction, mutant *A. nidulans* strains defective in certain defined steps of the Leloir and/or the oxido-reductive catabolic pathways were employed (see Table 1 for the genotype of the mutants). We hypothesized that induction would not occur in case the physiological inducer is formed by or downstream of the enzyme normally encoded by the mutated catabolic gene.

A frameshift mutation caused by a single base pair insertion near the 3′ end of the coding region of the galactokinase gene (*galE9*; Alam & Kaminskyj, 2013), either alone or in combination with a chromosomal translocation mutation disrupting the hexokinase gene (*hxkA1*; Flipphi *et al.*, 2003; and references therein), did not change the overall pattern of induction by D-galactose (Figs 2b and 3). Differences could, however, be seen in the time profile of transcript formation relative to the control (Fig. 2a), as expression consistently occurred earlier in the *galE* mutant than in the wild-type strain. In agreement with our previous results (Fekete *et al.*, 2012), except for a moderate expression on the pentose L-arabinose, no transcript was observed for any of the other monosaccharides tested in the reference strain (i.e. R21), and this was found to be the case in the *A. nidulans* mutant strains investigated in this study (data not shown).

The oxido-reductive pathway is also dispensable for *bgaD/lacpA* expression upon D-galactose induction

The mutation in the hexokinase (*hxkA1*) gene or a loss-of-function mutation in the L-arabinitol dehydrogenase gene (*araA1*) did not qualitatively change the expression of *bgaD* and *lacpA* upon D-galactose induction (Fig. 2c and d). These results suggested that D-galactose induction of the *bgaD-lacpA* gene cluster is mediated by either D-galactose itself or the first intermediate of the oxido-reductive catabolic pathway, galactitol (dulcitol).

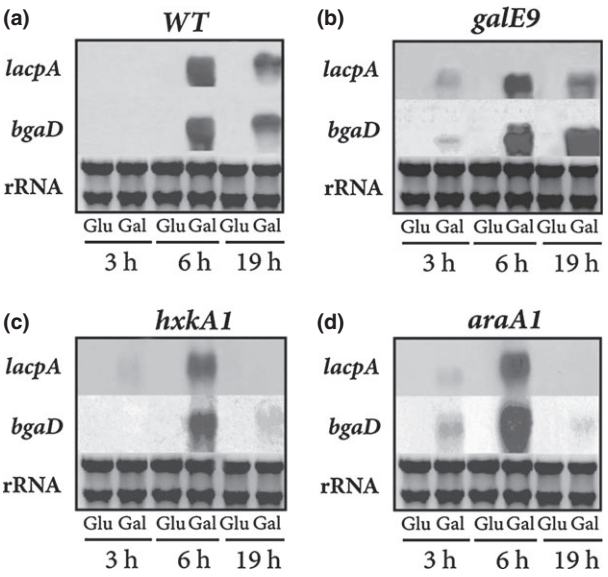

Fig. 2. Transcript analysis of the induction spectrum of the *bgaD-lacpA* gene cluster in response to D-glucose (Glu) and D-galactose (Gal) in various strains of *Aspergillus nidulans* (see Table 1 for a list of the mutants and their genotype). (a) Reference strain R21; (b) galactokinase mutant A214; (c) hexokinase mutant G092; (d) L-arabinitol dehydrogenase mutant G094. As a control for the quality and quantity of the loaded RNA, ribosomal RNAs (28S and 18S) were visualized with ethidium bromide; the negative of the original image is shown at the bottom (rRNA).

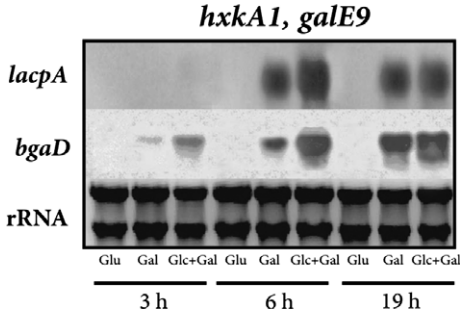

Fig. 3. Transcript analysis of the induction spectrum of the *bgaD-lacpA* gene cluster in the double galactokinase/hexokinase mutant EFES3 in response to D-glucose (Glu), D-galactose (Gal), or glycerol plus D-galactose (Glc + Gal). Similar to Fig. 2, the negative of the original image of the ribosomal RNAs (28S and 18S) is shown at the bottom (rRNA).

We therefore tested whether galactitol would be able to induce the formation of *bgaD and lacpA* transcripts as well as that of intracellular β-galactosidase activity (measured as the ability to hydrolyze ONPG).

Galactitol as a sole carbon source is able to support growth of *A. nidulans*, although maximal growth rates attainable are considerably lower than those on D-galactose (Fekete *et al.*, 2004; for quantitative data see Fig. 2d

of that paper). However, *bgaD* and *lacpA* transcription (Fig. 4) or bGal activity could not be detected at any time point during cultivation on the polyol as the sole carbon source (data not shown), neither in the wild-type reference strain (R21) nor in the galactokinase (*galE9*) mutant A214. Essentially identical results were obtained in medium replacement cultures (data not shown). We therefore concluded that upon growth on D-galactose, the formation of catabolic intermediates is not necessary for the induction of *bgaD* and *lacpA* genes to occur.

Discussion

In filamentous fungi, it is not uncommon that assimilation pathways are induced by a catabolic intermediate as the physiological inducer, rather than by the growth substrate itself. An unambiguous example in the model organism *A. nidulans* is the induction of ethanol catabolism (*alc* system) by acetaldehyde – the first pathway intermediate – where ethanol was shown to be inert (Flipphi *et al.*, 2001, 2002). Here, we investigated in *A. nidulans* whether for D-galactose induction of *bgaD* and *lacpA* – clustered genes that encode an intracellular beta-galactosidase and a lactose permease, respectively – downstream catabolism of D-galactose is dispensable or necessary. To provide experimental evidence, we employed mutant strains defective in the first step of the Leloir (galactose-1-kinase) and the second step of the oxido-reductive pathway (NAD-dependent galactitol dehydrogenase), respectively, and registered the presence or absence of transcripts of both genes consisting the gene cluster. Unfortunately, no *A. nidulans* mutants are known that are impaired in aldose 1-oxidoreductase, the activity believed to be responsible for the first step of the D-galactose oxido-reductive pathway (as well as of those by which the pentoses D-xylose and L-arabinose are catabolized). This may be due to the fact that there are at least two, but likely more, aldose 1-oxidoreductases with a wide substrate range present in ascomycete filamentous fungi (see Hasper *et al.*, 2000, for *Aspergillus niger*). In *A. nidulans*, there could be as many as twelve aldose 1-oxidoreductase genes specified in the genome (M. Flipphi, unpublished observations).

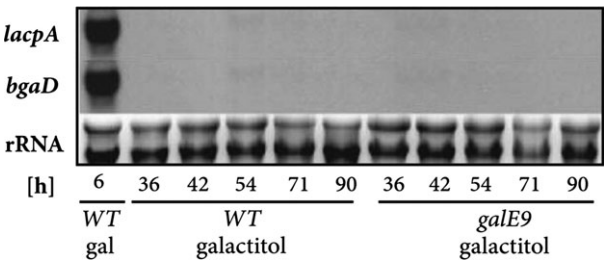

Fig. 4. Time profile of transcript formation of the *bgaD-lacpA* gene cluster in response to galactitol in submerged fermentation in the wild-type reference strain (R21) and the galactokinase mutant (A214). Strain R21 on D-galactose at 6 h after medium transfer (Gal) is shown as a control. The negative of the original image of the ribosomal RNAs (28S and 18S) is shown at the bottom (rRNA).

To bypass the unavailability of an *A. nidulans* mutant defective in the conversion of D-galactose into galactitol, we tested the inducibility of the two clustered genes on galactitol during induction experiments with pregrown mycelia and also during the course of fermentations in which galactitol was the sole carbon source. All data clearly showed that galactitol was unable to induce expression of *bgaD* and *lacpA*, independently of the stage of growth and despite the fact that the growth rate on galactitol is considerably lower than that on the monosaccharide. This observation is intriguing because these two genes are expressed constitutively to considerable levels in the carbon-derepressed background of the *creA*^{d}*30* mutant (Fekete *et al.*, 2012), while glucose repression was shown to be growth rate dependent in *A. nidulans*, that is, increasingly reduced with lower growth rates (Ilyés *et al.*, 2004). On the other hand, in mutants in the oxido-reductive pathway, that is, in L-arabinitol dehydrogenase or in hexokinase, the *bgaD/lacpA* gene cluster remained responsive to D-galactose, indicating that catabolic intermediates downstream of these enzymes play no role in the process of induction. An important conclusion is that galactitol was not converted back to D-galactose *in vivo* or 'under physiological conditions' as the polyol did not induce, not even in the *araA1* mutant which grows normally on D-galactose but does not grow on galactitol (De Vries *et al.*, 1994).

In a strain defective in the galactokinase step of the Leloir pathway, the *bgaD* and *lacpA* genes basically showed a similar expression pattern as the wild type, indicating that the Leloir pathway is likewise dispensable for the induction observed on D-galactose. Quantitative differences in the time profile of the induction are likely due to the variations in the growth rate. Indeed, both galactokinase (*galE9*) and L-arabinitol dehydrogenase (*araA1*) mutants have been documented to grow significantly slower on D-galactose than the wild-type strain (Fekete *et al.*, 2004). Moreover, the ability to grow on D-galactose appears irrelevant for the induction of *bgaD/lacpA* gene cluster to occur *per se*, as was proven by employing the double mutant EFES3 (Fekete *et al.*, 2004), defective in galactokinase and hexokinase. This *A. nidulans* double mutant cannot grow on D-galactose, but still produced *bgaD* and *lacpA* transcript upon transfer to medium with D-galactose being the only carbon source. Identical results were obtained on a mixed carbon source of D-galactose and glycerol – a neutral, that is, a noninducing, nonrepressing growth substrate – where car-

bon starvation was avoided (see Fig. 3). Altogether, our experiments clearly showed that D-galactose catabolism – either via the canonical Leloir pathway or the alternative oxido-reductive pathway – was not necessary for the induction of the *bgaD-lacpA* gene cluster to occur in the presence of D-galactose.

Induction by D-galactose in *A. nidulans* may involve GalX, a general regulator of galactose catabolism (Roberts, 1970; Christensen *et al.*, 2011). (NB. The *galX* gene corresponds with the classical *galA* locus). GalX is a structural ortholog of Gal4p and Lac9p zinc cluster activators of the *Saccharomyces cerevisiae* galactose catabolic pathway and the *K. lactis* lactose-galactose regulon, respectively (Christensen *et al.*, 2011; and references therein). On the other hand, in *A. nidulans*, induction by lactose was speculated to involve a regulatory gene that is functionally disrupted in strains carrying *lacA1*, a classical mutation that leads to the inability to grow on lactose without affecting growth on D-galactose (Roberts, 1963), as lactose uptake capacity was found essentially absent (Paszewski *et al.*, 1970). The fact that the *lacA1* mutation does not seem to interfere with D-galactose uptake (Fekete *et al.*, 2012) and its induction of ONPG-hydrolytic activity (Paszewski *et al.*, 1970; Gajewski *et al.*, 1972) might suggest that transcriptional induction of the *bgaD*/*lacpA* gene cluster by D-galactose and that by lactose are mediated by different, independent regulatory circuits, unlike the situation in the lactose-assimilating yeast *K. lactis* (Wray *et al.*, 1987). We are currently testing this hypothesis.

Acknowledgements

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