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Xyr1 receives the lactose induction signal and regulates lactose metabolism in *Hypocrea jecorina*

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Abstract **This study reports the vital regulatory influence of Xyr1 (xylanase regulator 1) on the transcription of hydrolytic enzyme-encoding genes and hydrolase formation on lactose in *Hypocrea jecorina*. While the transcription of the *xyr1* gene itself is achieved by release of carbon catabolite repression, the transcript formation of *xyn1* (xylanase 1) is regulated by an additional induction mechanism mediated by lactose. Xyr1 has an important impact on lactose metabolism by directly activating *xyl1* (xylose reductase 1) transcription and indirectly influencing transcription of *bga1* (β-galactosidase 1). The latter is achieved by regulating the conversion of D-galactose to the inducing carbon source galactitol.**

Keywords: Xyr1; Lactose; Xylanase; Cellulase; *Trichoderma reesei*; *Hypocrea jecorina*

1. Introduction

Hypocrea jecorina (anamorph *Trichoderma reesei* [1]) is a filamentous ascomycete which occurs in abundance wherever biomass is available. Breakdown of polysaccharides by *H. jecorina* is accomplished by a variety of hydrolytic enzymes synergistically working together [2,3]. Due to the high secretory capacity of this fungus (up to 100 g/L, [4]), in particular of its native enzymes, *H. jecorina* has gained industrial importance. Especially the xylanolytic and cellulolytic enzymes of *H. jecorina* are used in textile (e.g. [5]), food and feed (e.g. [6–8]) or paper industries (e.g. [9,10]).

It is therefore of particular importance to industry to have easily available and cheap inducers for the hydrolytic enzyme system of *H. jecorina*. Lactose had already been described to induce cellulase activity [11] some decades ago and since then its activating effect on cellulase formation or on transcription of cellulolytic enzyme-encoding genes has been reported several times (e.g. [12–15]). Obviously, lactose (1,4-*O*-β-D-galactopyranosyl-D-glucose) does not represent a natural substrate for *H. jecorina,* as it is the predominant carbohydrate in milk and dairy products. This fact leads to the question whether lactose acts as a real inducer for the formation of hydrolytic enzymes since the mechanism involved in cellulase and xylanase activation could also be a release from carbon catabolite repression (ccr) as previously suggested [16].

Lactose is cleaved into D-galactose and D-glucose by a β-galactosidase (EC 3.2.1.23). In *H. jecorina* this is mainly encoded by the *bga1* gene [17]. The α-anomer of galactose is then specifically metabolized by galactokinase (EC 2.7.1.6, encoded by *gal1*, [15]) to give D-galactose-1-phosphate [18] which then enters the Leloir pathway [19]. The first step of an alternative pathway to the Leloir pathway [15,20,21] was described to be catalyzed by D-xylose reductase (encoded by *xyl1*) [20]. Xyl1 was proposed to reversibly reduce D-galactose to galactitol, which is further catabolized to sorbose and fructose which then enters glycolysis. Recently, we have demonstrated that in *H. jecorina* the activator Xyr1 (Xylanase regulator 1) is essential for the activation of *xyl1* transcript formation and regulation of D-xylose reductase expression [22]. Furthermore, it has been reported that Xyr1 is indispensable for transcriptional regulation of the major hydrolytic enzyme-encoding genes, e.g. *cbh1* and *cbh2* (cellobiohydrolases I and II, EC 3.2.1.- and EC 3.2.1.91, respectively), *egl1* (*endo*-β-1,4-glucanase I, EC 3.2.1.4), *xyn1* and *xyn2* (*endo*-β-1,4-xylanase I and II, EC 3.2.1.8), *bxl1* (β-xylosidase I, EC 3.2.1.37) as well as *bgl1* (1,4-β-glucosidase I, EC 3.2.1.21) [22]. Xyr1 mediates the induction of gene expression of the above mentioned genes regardless of the mode of gene expression (basal, derepressed or induced) or of the applied inducer (xylose, xylobiose, or sophorose) [22].

Here, we report for the first time the predominant influence of Xyr1 on both hydrolytic enzyme activity formation and regulation of transcription of major hydrolytic enzyme-encoding genes (*cbh1*, *cbh2*, *xyn1* and *xyn2*) on lactose. Furthermore, comparison of *xyr1* transcript levels of a Cre1-negative strain [23] with the wild-type cultivated on glucose and lactose revealed *xyr1* transcription on lactose to be governed by a derepression mechanism. In contrast to *xyr1* transcript formation, transcription of the *xyn1* gene is additionally subject to a lactose specific induction mechanism different from the xylose-mediated induction. Finally, Xyr1 was identified to regulate the first step in the alternative galactose pathway (*vide supra*) by directly activating *xyl1* transcription. But Xyr1 does not influence the expression of galactokinase, leading into the Leloir pathway. Thus, it appears that Xyr1 is an indispensable transcription factor for expression of genes that encode hydrolytic enzymes on lactose and plays an

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important role in the regulation of the lactose metabolism of *H. jecorina*.

2. Materials and methods

2.1. Strains and growth conditions

H. jecorina (*T. reesei*) QM9414 (parental strain; ATCC 26921), a *xyr1* deletion strain (Δ*xyr1*-strain) [22], Rut-C30, a Cre1 negative strain, (ATCC 56765) [24] and CK11, a *cre1* retransformation strain of Rut-C30 [25] were used throughout this study.

Direct cultivations were performed by adding a suspension of spores directly to a medium containing the carbon source of interest. They were carried out threefold in 1-L-Erlenmeyer flasks with 200 mL Mandels–Andreotti medium (MAm) [26] containing 1% (w/v) xylose, glucose or lactose and inoculated with 10^8 conidia per litre (final concentration). Incubation was continued for up to 75 h at 30 °C and 250 rpm.

For replacement experiments strains were pre-grown first and mycelia then put into media containing the carbon source of interest. Thus, respective strains were pre-cultured in 1-L-Erlenmeyer flasks on a rotary shaker (250 rpm) at 30 °C for 18 h in 250 mL of MAm applying 1% (w/v) glycerol as carbon source. 10^8 conidia per liter (final concentration) were used as inoculum. Pre-grown mycelia were washed and equal amounts resuspended in MAm containing 1% (w/v) galactitol or glucose as carbon source or in MAm without a carbon source (control). Incubation was continued for 3 or 6 h if cultivated on galactitol or glucose respectively and for 3 h if cultivated on medium without carbon source. Threefold biological replicates were performed.

2.2. Enzyme assays

Xylanase and cellulase activities were measured applying Xylazyme AX or Cellazyme C Tablets, respectively (Megazyme, Wicklow, Ireland), according to the manufacturer's instructions (40 °C for 10 min). One Unit of activity is defined as the amount of enzyme required to release one micromole of xylose or glucose reducing-sugar-equivalents per minute.

2.3. Quantitative RT-PCR

Parallel DNA- and RNA-extraction and reverse transcription was performed as described previously [22]. PCRs were performed in an iCycler iQ, Real-Time Detection System (Biorad, Herkules, US). Taqman assays for monitoring *xyn1*, *xyn2*, *cbh1,* and *cbh2* transcript levels were carried out as published elsewhere [22]. For *xyr1*, *xyl1*, *gal1* and *bga1* transcription analysis, a SYBR Green assay with reference to actin was performed including 1× iQ SYBR Green Supermix (Biorad), 0.1 μM forward primer, 0.1 μM reverse primer, and cDNA as template. Primers and probes are given in Table 1. The following PCR program was used: 3 min initial denaturation at 95 °C, followed by 45 cycles of 15 s at 95 °C, 15 s at 59 °C (at 60 °C for *xyr1*) and 15 s at 72 °C. Calculations of the amounts of mRNA (cDNA) per gene dose were performed as described elsewhere [22]. Results of transcription analysis are given in transcripts per gene dose if referenced to DNA and in relative transcript ratio if referenced to transcription of the actin gene. The values given in the figures are means of three independent experiments. Error bars indicate standard deviations.

3. Results and discussion

3.1. Hydrolase expression in a xyr1 deletion strain on lactose

Xyr1 was reported to be the general activator of hydrolytic genes regardless of the inducing substance or activating carbon source *H. jecorina* was cultivated on, e.g. xylan, cellulose, xylobiose, xylose or sophorose [22]. To investigate if the lactose signal is also mediated by Xyr1, the *H. jecorina* QM9414 strain and a Δ*xyr1*-strain were cultivated on lactose-containing medium. Neither xylanase activity (Fig. 1A) nor cellulase activity (Fig. 1B) could be detected in the Δ*xyr1*-strain. The effect of Xyr1 was verified by comparing the transcript levels of the major xylanase and cellulase-encoding genes *xyn1*, *xyn2, cbh1* and *cbh2*. Transcripts from all those genes are formed by the wild-type when grown on lactose, which is in complete accordance with previously published results [12,13]. Nonetheless, we did not detect any *xyn1*-, *cbh1*- and *cbh2*-mRNA and only a strikingly reduced *xyn2* transcript from the Δ*xyr1*-strain (Fig. 1C). It shall be noted that the absence of Xyr1 had a strong impact on the growth of *H. jecorina* on lactose during the late stages of cultivation, as biomass formation in the Δ*xyr1*-strain dropped after 40 h of cultivation (*vide infra*). Nevertheless, this is not the reason for the absence of transcripts since samples for quantitative RT-PCR analysis were taken during the early growth period of both strains when they exhibited similar biomass formation (data not shown).

Thus, we conclude that Xyr1 receives the lactose signal and subsequently mediates transcriptional regulation of hydrolase formation.

3.2. Carbon catabolite derepression is involved in regulating xyr1 transcript formation on lactose

As lactose affects transcription of hydrolytic genes as well as hydrolase expression and growth behavior of a Δ*xyr1*-strain we became interested in evaluating its impact on *xyr1* transcript formation. We therefore compared *xyr1* transcript levels of the wild-type after cultivation on glucose and lactose. We found higher amounts of *xyr1* transcript on lactose (Fig. 2A) which can either be due to induction mechanisms or to a release from ccr. To find out which of these mechanisms is involved, we cultured a Cre1 negative strain (Rut-C30) and – to control if the observed effect is due to a lack of Cre1 – a corresponding *cre1* retransformation strain (CK11) on both carbon sources. Similar levels of *xyr1* transcripts were obtained (Fig. 2B) suggesting that derepression and not induction causes the detected *xyr1* transcription levels on lactose.

As the regulatory mechanism of *xyr1* transcription is derepression, we also asked ourselves the question whether mRNA formation of hydrolases-encoding genes on lactose is due to

Table 1
Primers and probes used throughout the study

Name	Sequence (5′–3′)	Employment[a]
actf	TGAGAGCGGTGGTATCCACG	Actin real-time PCR
actr	GGTACCACCAGACATGACAATGTTG	Actin real-time PCR
bga1f	CGTTTGATCCTTTCGGCGGCT	*bga1* real-time PCR
bga1r	CCAAAGGTCATGTATATGTTGAAGATGGTC	*bga1* real-time PCR
gal1f	GGAGGCATGGACCAGGC	*gal1* real-time PCR
gal1r	GACATGCTTGTTGGAGGTGACG	*gal1* real-time PCR
xorf	CTGTGACTATGGCAACGAAAAGGAG	*xyl1* real-time PCR
xorr	CACAGCTTGGACACGATGAAGAG	*xyl1* real-time PCR

[a]Employment of the oligonucleotides during the study is given.

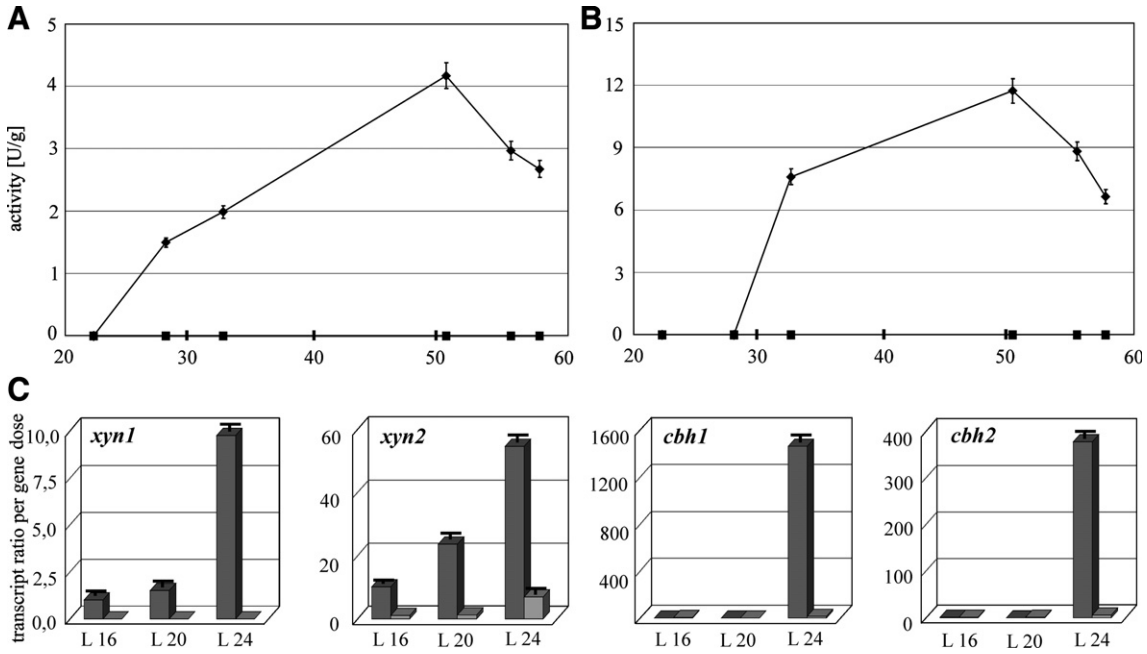

Fig. 1. Cultivation of the parental strain QM9414 and of a Δ*xyr1*-strain on MAm supplemented with 1% (w/v) lactose (L) as sole carbon source at 30 °C. Data are means of results from three independent biological replicates. Error bars indicate standard deviations. Xylanase activities (A) and cellulase activities (B) of the parental strain QM9414 (diamonds) and a Δ*xyr1*-strain (squares). (C) Relative transcription levels of *xyn1*, *xyn2*, *cbh1* and *cbh2* of the parental strain QM9414 (dark grey) and the Δ*xyr1*-strain (light grey). Samples were taken after 16, 20 and 24 h of cultivation.

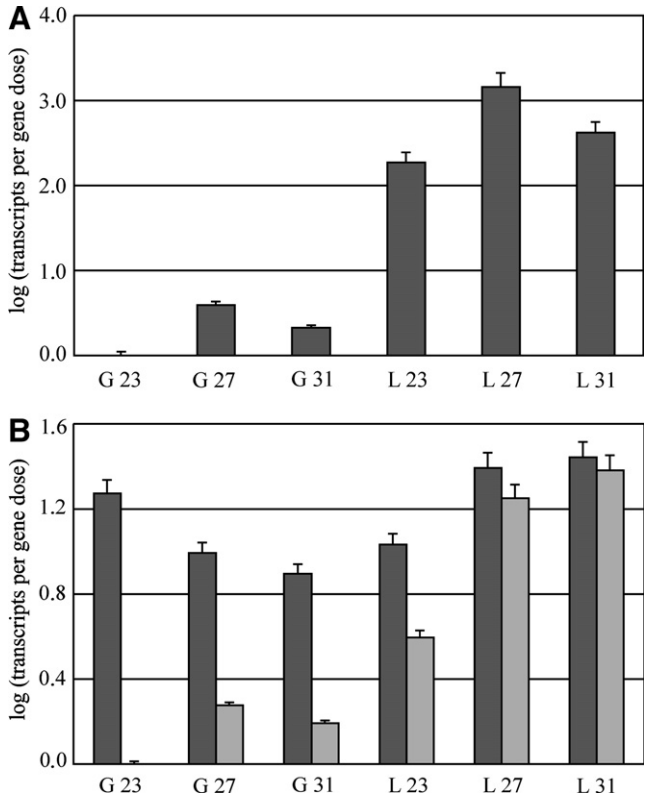

Fig. 2. Relative transcription levels of *xyr1*. The parental strain QM9414 (A) as well as the Rut-C30 (dark grey) strain and CK11 (light grey) strain (B) were cultured on MAm containing 1% (w/v) glucose (G) or lactose (L) as sole carbon source and samples were taken after 23, 27 and 31 h, respectively.

such derepression or due to induction. *Xyn1* transcription is well known to be affected by Cre1-mediated ccr/derepression [16], and therefore it is an ideal model gene to answer this question. Hence, the amounts of transcript of *xyn1* were analyzed in the Rut-C30 strain cultivated on glucose, lactose or xylose. While there is only a small amount of transcript formed on glucose due to release from ccr [16], significantly more *xyn1* transcript could be detected on lactose, but still not reaching by far mRNA levels produced on xylose (Fig. 3), previously described to strongly induce *xyn1* transcript formation [16,27]. Obviously, *xyn1* transcription on lactose is not only governed by a Cre1 dependant release from ccr but also by a lactose specific induction mechanism, although distinct from the one provoked by xylose (Fig. 3). Summarizing, it can be said that *xyn1* transcription in *H. jecorina* is inducible by lactose, the induction signal is mediated by Xyr1 but the mechanism is not the same as that triggered by xylose. As mentioned before, lactose does not occur in the natural environment of *H. jecorina*. Presumably, galactan side chains of pectins and arabinogalactans, which are carbohydrate components of plant cell walls ([28,29] and citations therein), are the original substrates of β-galactosidase of which it releases terminal galactose residues [30,31].

3.3. Regulatory impact of Xyr1 on H. jecorina lactose metabolism

As demonstrated so far, Xyr1 is indispensable for hydrolase expression on lactose in *H. jecorina*. To elucidate the impact of Xyr1 on the metabolism of lactose, transcription of several involved genes encoding, i.e. β-galactosidase (*bga1*), D-xylose reductase (*xyl1*) and galactokinase (*gal1*) was examined. A schematic presentation of the lactose metabolism in *H. jecorina* is given in Fig. 4A. It was reported that *bga1* transcripts are

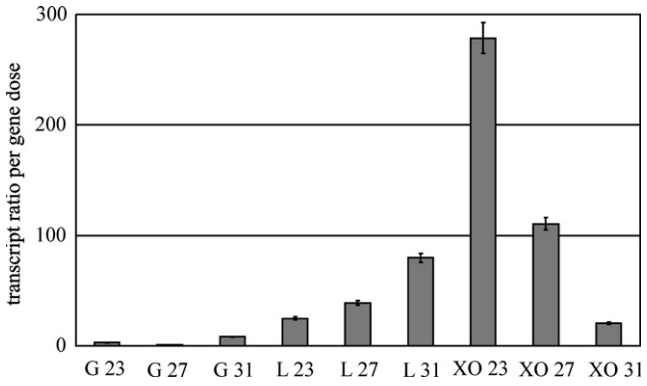

Fig. 3. Relative transcription levels of *xyn1*. The Rut-C30 strain was cultured on MAm containing 1% (w/v) glucose (G), lactose (L) or xylose (XO) as sole carbon source and samples were taken after 23, 27 and 31 h, respectively.

formed if the wild-type is cultivated on lactose and that *bga1*-encoded β-galactosidase activity plays a role in cellulase production on lactose [17]. Thus, we cultivated a wild-type and a Δ*xyr1*-strain on lactose and measured *bga1* transcript formation. We found reduced *bga1* transcript levels in the Δ*xyr1*-strain compared to the wild-type (Fig. 4B) pointing at an involvement of Xyr1 in the regulation of *bga1* transcription.

Recently, we have reported on the involvement of Xyr1 in the D-xylose metabolism of *H. jecorina*, namely its function as the main activator of D-xylose reductase expression [22]. As this D-xylose reductase was described to catalyze the first step of an alternative pathway [15,20,21] to the Leloir pathway [19], we checked *xyl1* transcript formation on lactose in a Δ*xyr1*-strain compared to the wild-type. Since, we detected strongly reduced *xyl1* transcript levels in the mutant strain we could confirm the ability of Xyr1 to regulate D-xylose reductase, even if cultivated on lactose (Fig. 4C). In silico analysis of the *xyl1* promoter region resulted in the identification of two Xyr1 binding domains (GGCTAA [27]) within 1.4 kb upstream of the *xyl1* structural gene and thus confirms the above observed results.

In order to investigate the influence of Xyr1 on the first step of the Leloir pathway, we compared *gal1* transcription levels of a Δ*xyr1*-strain to those of the wild-type, both cultivated on lactose. Since we found similar transcription levels in both strains (Fig. 4D), we concluded that Xyr1 does not influence this catabolic pathway, at least not at that stage. But as already mentioned, galactokinase specifically metabolizes only the α-anomer of galactose. Because of the time needed for non-enzy-

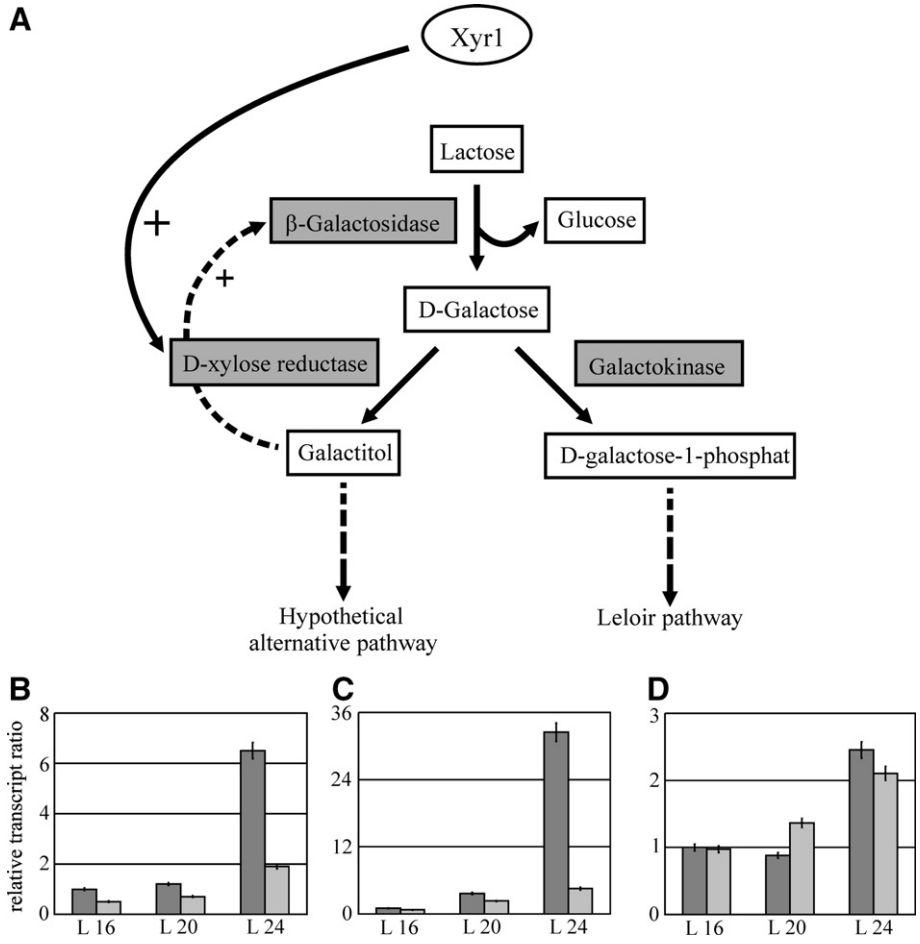

Fig. 4. (A) Schematic presentation of the lactose metabolism in *H. jecorina*. Involved enzymes of both possible pathways are given in dark grey boxes and respective intermediates are given in white boxes. The activating influence (+) of Xyr1 or galactitol, respectively is indicated by an arrow. Relative transcription levels of *bga1* (B), *xyl1* (C) and *gal1* (D) of the parental strain QM9414 (dark grey) and of the Δ*xyr1*-strain (light grey). Samples were taken after 16, 20 and 24 h cultivation on MAm supplemented with 1% (w/v) lactose (L) as sole carbon source.

matic mutarotation (at 30 °C, pH 6.5–7.0) is quite long (some hours) [32], it suggests itself that an accumulation of toxic levels of the β-anomer in the cell occurs [33]. Thus, the Δ*xyr1*-strain may be expected to show strongly reduced growth on galactose derived from lactose metabolism because this strain can make only restricted use of the second pathway alternatively to the slowly metabolizing Leloir pathway. This assumption is in good accordance with *vide supra* reported decline in biomass formation of the Δ*xyr1*-strain during late stages of cultivation on lactose. Furthermore, this strain shows similar growth on agar plates containing galactose compared to the wild type (data not shown), most probably due to the fact that synthetic galactose is a mixture of the α- and the β-anomer.

3.4. Influence of galactitol on lactose metabolism in H. jecorina

As published recently, galactitol mediates the induction of extracellular β-galactosidase [20] and, as reported in this study, Xyr1 regulates *xyl1* transcription on lactose (*vide supra*). Both facts taken together stress the question whether Xyr1 directly regulates *bga1* transcription or if its impact is indirect via provision of galactitol by activation of the D-xylose reductase activity. To determine if the influence of Xyr1 on *bga1* transcription is indirect, we replaced the Δ*xyr1*-strain and the wild-type on medium containing galactitol as sole carbon source. Transcription levels of *bga1* in both strains were similar

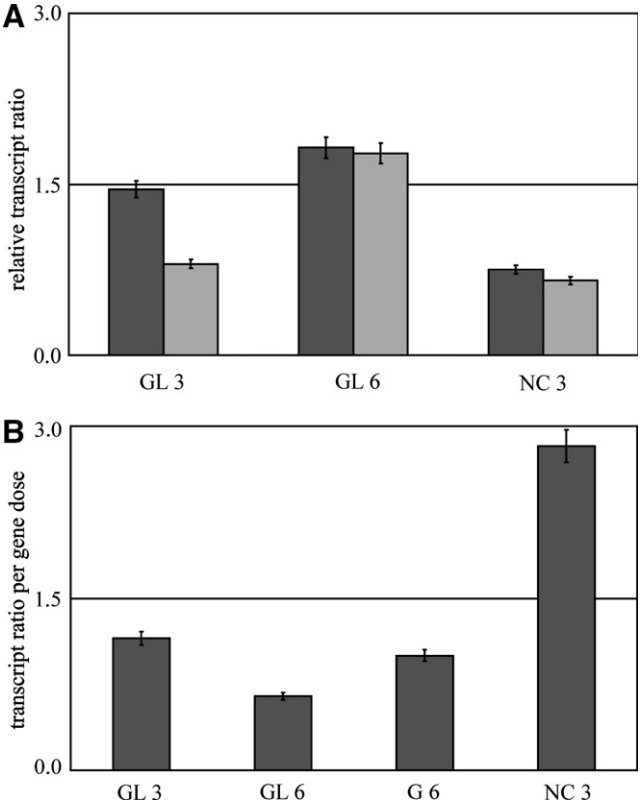

Fig. 5. (A) Relative transcription levels of *gal1* of the parental strain QM9414 (dark grey) and of the Δ*xyr1*-strain (light grey). Both strains were pre-cultured on glycerol and thereafter transferred to MAm containing 1% (w/v) galactitol (GL) as sole carbon source, or without carbon source (NC), and incubated for 3 and 6 or 3 h, respectively. (B) Relative transcription levels of *xyr1* of the parental strain QM9414. A pre-culture on glycerol was transferred to MAm containing 1% (w/v) galactitol (GL) or glucose (G) as sole carbon source, or without carbon source (NC), and incubated for 3 and 6 or 3 h, respectively.

(Fig. 5) suggesting that Xyr1 is not involved in the direct induction of *bga1* transcription on galactitol. Consequently, we analyzed transcription of the *xyr1* gene of the wild-type replaced on galactitol, glucose or medium without carbon source. As *xyr1* transcription levels on galactitol were lower than on a medium without carbon source (Fig. 5B), we can rule out that transcript formation of *xyr1* is induced by galactitol. We already found strong evidence for a Cre1-dependent ccr/derepression mechanism on glucose of *xyr1* transcription (*vide supra*, Fig. 2B). *Xyr1* transcript formation is obviously also repressed on galactitol since the transcript levels are quite similar to the ones on glucose (Fig. 5B).

In accordance to these results, in silico analysis of the *bga1* 5′ upstream region revealed no putative Xyr1 binding domain within the *bga1* promoter. These data indicate an indirect influence of Xyr1 on the activation of *bga1* transcription by making galactitol which acts as the actual inducer available.

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