

ORIGINAL ARTICLE

# Extra- and intracellular lactose catabolism in *Penicillium chrysogenum*: phylogenetic and expression analysis of the putative permease and hydrolase genes

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*Penicillium chrysogenum* is used as an industrial producer of penicillin. We investigated its catabolism of lactose, an abundant component of whey used in penicillin fermentation, comparing the type strain NRRL 1951 with the high producing strain AS-P-78. Both strains grew similarly on lactose as the sole carbon source under batch conditions, exhibiting almost identical time profiles of sugar depletion. *In silico* analysis of the genome sequences revealed that *P. chrysogenum* features at least five putative  $\beta$ -galactosidase (bGal)-encoding genes at the annotated loci Pc22g14540, Pc12g11750, Pc16g12750, Pc14g01510 and Pc06g00600. The first two proteins appear to be orthologs of two *Aspergillus nidulans* family 2 intracellular glycosyl hydrolases expressed on lactose. The latter three *P. chrysogenum* proteins appear to be distinct paralogs of the extracellular bGal from *A. niger*, LacA, a family 35 glycosyl hydrolase. The *P. chrysogenum* genome also specifies two putative lactose transporter genes at the annotated loci Pc16g06850 and Pc13g08630. These are orthologs of paralogs of the gene encoding the high-affinity lactose permease (*lacpA*) in *A. nidulans* for which *P. chrysogenum* appears to lack the ortholog. Transcript analysis of Pc22g14540 showed that it was expressed exclusively on lactose, whereas Pc12g11750 was weakly expressed on all carbon sources tested, including D-glucose. Pc16g12750 was co-expressed with the two putative intracellular bGal genes on lactose and also responded on L-arabinose. The Pc13g08630 transcript was formed exclusively on lactose. The data strongly suggest that *P. chrysogenum* exhibits a dual assimilation strategy for lactose, simultaneously employing extracellular and intracellular hydrolysis, without any correlation to the penicillin-producing potential of the studied strains. *The Journal of Antibiotics* (2014) 67, 489–497; doi:10.1038/ja.2014.26; published online 2 April 2014

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## INTRODUCTION

The heterodisaccharide lactose (1,4-O- $\beta$ -D-galactopyranosyl-D-glucose) occurs mainly in milk where it makes up 2–8% of the dry weight.<sup>1</sup> In mammals, it is formed by the action of lactose synthetase, which transfers the galactosyl unit from UDP-galactose to D-glucose.<sup>2</sup> In the intestine, lactose is hydrolyzed by membrane-anchored lactase/phlorizin hydrolase (lactose galactohydrolase; EC 3.2.1.108), a family 35 glycosyl hydrolase (GH).<sup>3</sup> Whey, the lactose-rich by-product of cheese manufacture, has long been used as a cheap growth substrate for the production of valuable metabolites such as penicillin or (hemi)cellulolytic enzymes by filamentous fungal cell factories.<sup>4,5</sup> The strong, lactose-inducible cellulase gene promoters from *Trichoderma reesei* are also used for commercial recombinant protein production.<sup>6</sup>

Two strategies have been described for the catabolism of lactose in fungi: extracellular hydrolysis and subsequent uptake of the resulting monomers, that is, D-glucose and D-galactose, and uptake of the disaccharide followed by intracellular hydrolysis.<sup>7</sup> Some well-studied ascomycetes such as *Kluyveromyces lactis*<sup>8–10</sup> and *Aspergillus nidulans*<sup>11</sup> possess an intracellular pathway for lactose assimilation, whereas in *A. niger*,<sup>12,13</sup> lactose hydrolysis occurs extracellularly. Fungal  $\beta$ -galactosidases (bGals) ( $\beta$ -D-galactoside galactohydrolase; EC 3.2.1.23) can be distinguished into extracellular enzymes that are characterized by an acidic pH optimum, and into intracellular ones that optimally function at neutral pH. The former generally belong to glycosyl hydrolase family 35 (GH35;<sup>14</sup>), whereas the latter usually are GH2 proteins, although some  $\beta$ -glucosidases (GH1) also act on lactose.<sup>15</sup> In *Neurospora crassa*, both bGal isozymes are produced on

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This paper is dedicated to the memory of Dr Ferenc Sztaricskai (1934–2012), distinguished Professor of Organic Chemistry at the University of Debrecen and former Editor of *The Journal of Antibiotics*.

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lactose medium.<sup>16</sup> It has to be noted that in the past, both types of activity were commonly studied using artificial chromogenic substrates, such as *O*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) or *P*-nitrophenyl- $\beta$ -D-galactopyranoside, rather than lactose.

Strains of *P. chrysogenum* (species complex *P. chrysogenum sensu lato*, phylogenetic species *Penicillium rubens*<sup>17</sup>) are used as industrial producers of penicillin and structurally related antibiotics. As such, *P. chrysogenum* is amongst the most important fungi employed in the biotech industry, with thousands of studies devoted to its potential to produce natural and semisynthetic antibiotics.<sup>18–20</sup> However, apart from traits directly related to penicillin biosynthesis, general aspects of their carbon metabolism have received little attention. In particular, its utilization of lactose and that of its monomer product D-galactose have never been studied in depth, which is surprising, given the scale on which penicillin has been produced on whey as the growth substrate for decades.

Formation of an intracellular hydrolase activity against ONPG has been described in the industrial *P. chrysogenum* strain NCAIM 00237, and the corresponding enzyme was partially characterized.<sup>21,22</sup> Unpublished data (Biro and Szentirmai, personal communication) suggested that the observed activity may be a side activity of a mycelium-associated enzyme with a principal *N*-acetylglucosaminidase activity.<sup>23,24</sup> On the other hand, extracellular bGal has been reported in related species, for example, in *P. canescens*<sup>25</sup> and *P. simplicissimum*.<sup>26</sup>

The goal of the present study was to identify genes coding for the structural activities (that is, enzymes and transporters) putatively involved in lactose catabolism in the wild-type reference strain NRRL 1951 as well as the industrial penicillin producer AS-P-78.

## MATERIALS AND METHODS

### Strains and cultivation conditions

Two strains of *P. chrysogenum* (*sensu lato*) were used in this work. NRRL 1951 was isolated from nature<sup>27</sup> and is the parent strain from which the large majority of improved penicillin producers are derived.<sup>28</sup> One of those, the increased-titer strain AS-P-78<sup>29</sup> was a kind gift from Antibióticos S.A. (León, Spain). Minimal medium for shake flask and bioreactor cultivations were formulated as described previously.<sup>30</sup> All growth media inoculated with pregrown mycelia were completely synthetic, whereas 0.01% (w/v) peptone was included in media that were inoculated with conidia (see the Results section for explanation). Carbon sources (that is, sugars or glycerol) were used at concentrations up to 1.5% (w/v). Supplements were added from sterile stock solutions. Shake flask cultures were incubated at 28 °C in 500-ml Erlenmeyer flasks in a rotary shaker at 200 r.p.m. Where appropriate, cultures were inoculated with  $5 \times 10^6$  *P. chrysogenum* conidia per milliliter of medium.

For induction experiments, replacement cultures were used for which mycelia were pregrown for 48 h in minimal medium containing 1% (v/v) glycerol as the carbon source, and harvested by filtration on a sintered glass funnel. After a thorough wash with cold sterile water, biomass was transferred to flasks with fresh minimal medium containing a range of concentrations (from 0.1 to 25 mM) of the various carbon sources tested. Samples were taken after 4 h of further incubation to assess inductory ability. Preliminary trails had established that 4 h of contact is the time lapse in which maximal induced activity levels were achieved, with a minimal variation in the biomass concentration. For transcript analysis, samples were taken 4 and 8 h after the transfer of mycelia.

Bioreactor cultures were inoculated with the harvested and washed biomass of 200 ml minimal medium/glycerol-grown cultures. Fermentations were carried out in a 9-l glass bioreactor (Inel, Budapest, Hungary) with a culture (working) volume of 6 l, equipped with one six-blade Rushton disc turbine impeller. Operating conditions were pH 6.0, 28 °C and 0.5 v.v.m. (volumes of air per volume of liquid per minute). Dissolved oxygen levels were maintained at 20% saturation and were controlled by means of the agitation rate. To minimize medium loss, the waste gas (from the headspace) was cooled in a reflux condenser connected to an external cooling bath (4 °C) before exiting the system.

The yield coefficient ( $Y_{x/s}$ ) was calculated as the ratio of the maximal concentration of biomass achieved during fermentation and the initial carbon source concentration. Specific growth rates ( $\mu$ ; h<sup>-1</sup>) were calculated from the increased dry cell weight over the time lapsed until carbon source exhaustion.<sup>31</sup>

All chemicals used were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA).

### Genomic DNA and total RNA isolation

Mycelia were harvested by filtration over nylon mesh and thoroughly washed with sterile distilled water. Excess liquid was removed by squeezing between paper sheets and the biomass was quickly frozen in liquid nitrogen. For nucleic acid isolation, frozen biomass was ground to dry powder using a liquid nitrogen-chilled mortar and pestle. Genomic DNA was extracted using Promega's Wizard SV Genomic DNA Purification System, whereas total RNA was isolated with Promega's SV Total RNA Isolation System (Promega, Fitchburg, WI, USA).

### Northern blot and reverse transcriptase-PCR (RT-PCR) analysis

Standard procedures<sup>32</sup> were applied for the quantification, denaturation, gel separation and nylon blotting of total RNA, and the subsequent hybridization of the resultant membranes with gene-specific probes (Table 1). Agarose gels were charged with 5  $\mu$ g RNA per slot. Probes were digoxigenin-labeled using the PCR DIG Probe Synthesis Kit (Roche Applied Science, Penzberg, Germany) primed with gene-specific oligonucleotides (listed in Table 1) off wild-type (NRRL 1951) genomic DNA. Gene-specific hybridization was visualized with a Lumi-Film Chemiluminescent Detection film (Roche Applied Science). All transcript analyses were independently repeated at least twice. For

**Table 1 Primers used for the amplification of the putative  $\beta$ -galactosidase and lactose permease genes in *P. chrysogenum***

Given name	Putative activity function	Gene ID	Oligonucleotide sequence (5'–3')	Amplicon size, (bp)
<i>bgaA</i>	Extracellular $\beta$ -galactosidase	Pc16g12750	Pc16g12750F: AACTCTGCCTACAACACTACTG Pc16g12750R: TCTCATACTTAGGCTGGTC	1.300
<i>bgaB</i>	Extracellular $\beta$ -galactosidase	Pc06g00600	Pc06g00600F: TACTCGGCACCAATCTCAG Pc06g00600R: AGCCCAGAAATCATAACGC	639
<i>bgaC</i>	Extracellular $\beta$ -galactosidase	Pc14g01510	Pc14g01510F: TAAGAAGACAGCCTACGG Pc14g01510R: TCTTGGACCCTTTGTATC	665
<i>bgaD</i>	Intracellular $\beta$ -galactosidase	Pc22g14540	Pc22g14540F: ACGGTAGAGAGCAACAGCC Pc22g14540R: GAGACCATCCATCACAACCG	1.080
<i>bgaE</i>	Intracellular $\beta$ -galactosidase	Pc12g11750	Pc12g11750F: CTCTTAAACTGGAACACC Pc12g11750R: TCCAGACTCCATCAACAC	809
<i>lacA</i>	Lactose permease	Pc13g08630	Pc13g08630F: GCAAGACAAGAAGGCACAAG Pc13g08630R: TTTCAACGGCATAGGCCAG	800
<i>lacB</i>	Lactose permease	Pc16g06850	Pc16g06850F: GGATGTCTGAAATACCAAAAG Pc16g06850R: GCGAAGAAGTAGATGAACAC	930

RT-PCR analysis of Pc12g11750 (= *bgaE*), first-strand complementary DNA (cDNA) was synthesized by a RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA), according to the manufacturer's protocol. cDNA was subsequently used as a template for PCR employing the same gene-specific primer pair as for Northern analysis (Table 1). PCR was performed in a volume of 25  $\mu$ l containing 4  $\mu$ l of cDNA, and Dream Taq polymerase (Thermo Scientific). Cycling conditions after an initial denaturing at 95 °C for 2 min were: 40 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min and a final step of 72 °C for 5 min. The gene encoding the translation elongation factor- $\alpha$  (*tef1*) was used as an expression control.<sup>33</sup>

### Bioinformatics

The published *P. chrysogenum* whole-genome sequences<sup>34</sup> are from the low-titer laboratory strain Wisconsin 54–1255, a direct descendent of NRRL 1951. The fungal, whole-genome shotgun contig and non-redundant nucleotide (nt/nr) databases of the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) were screened with TBLASTN<sup>35</sup> using *Aspergillus* bGal (glycosyl hydrolase families 2 or 35) and lactose permease proteins as queries, and gene models were manually deduced from selected genomic DNA sequences. These searches included the recently published genome sequences from four additional species of the *Penicillium* genus: *P. paxilli* ATCC 26601 (NCBI whole-genome shotgun sequencing project number AOTG01000000); *P. decumbens* (AGIH000000000 and GII00000000); *P. digitatum* (AKCT000000000 and AKCU000000000); and *P. chrysogenum sensu strictu* (<http://genome.jgi.doe.gov/Pench1/Pench1.home.html>).<sup>17</sup> For phylogenetic analysis, protein sequences were aligned using CLUSTALW,<sup>36</sup> and then edited manually with GeneDock.<sup>37</sup> A phylogenetic tree was reconstructed feeding the alignment into MEGA-5 software<sup>38</sup> using the neighbor-joining algorithm<sup>39</sup> with the Jones–Taylor–Thornton model. Stability of clades was evaluated by 500 bootstrap rearrangements.

### Analytical methods

Mycelial dry cell weight was determined from 10 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. Dry weight data reported in the Results section are the average of the two separate measurements, which never deviated > 14%. The concentration of D-glucose, D-galactose, L-arabinose, D-xylose, glycerol and lactose in growth media was determined by HPLC with a proton exchange column (Bio-Rad Aminex HPX–87H<sup>+</sup>, Bio-Rad, Hercules, CA, USA) thermostated at 55 °C, using isocratic elution with 10 mM H<sub>2</sub>SO<sub>4</sub> and refractive index detection. The concentrations given are the average of two independent measurements, which never deviated > 3%.

### Preparation of cell-free extracts and medium samples

To obtain a cell-free extract, 10 ml of culture broth were withdrawn, suction filtered and the harvested mycelia thoroughly washed with 0.1 M sodium phosphate buffer, pH 7.0. The biomass was then resuspended in 5 ml of the same buffer, and homogenized in a pre-cooled Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 3000 g (15 min, 4 °C) and the supernatant was immediately used to assay intracellular  $\beta$ -galactosidase/lactase activity. To determine extracellular  $\beta$ -galactosidase/lactase activity, 5 ml of culture broth were withdrawn, the mycelia spun down (3000 g for 15 min) and the supernatant was directly assayed.

### Hydrolase assays

Intracellular bGal activity was assayed by incubating a mixture of 0.5 ml of cell-free extract and 0.5 ml of a 6 mM ONPG solution for 30 min at 28 °C. Reactions were terminated by the addition of 2 ml 1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> and the OD<sub>410</sub> was determined using an Amersham photospectrometer (Amersham, UK). For each extract, a sample to which the Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> was added before the ONPG substrate provided a blank measure.

Extracellular activity was assayed similarly except that a mixture of 0.5 ml of cell-free medium supernatant and an equal volume of a 6-mM ONPG solution in 0.1 M sodium phosphate buffer (pH 5.0) was used.

One unit (U) of  $\beta$ -galactosidase activity corresponds to the release of 1  $\mu$ mol of O-nitrophenol per minute, using a concentration calibration curve determined under the conditions of the assay. Cell-free extract and medium protein contents were determined with a modified Lowry method,<sup>40</sup> using bovine serum albumin for calibration. Specific activity was expressed as U mg<sup>-1</sup> protein.

Lactose-hydrolyzing (lactase) activity was determined by incubating at 28 °C for 30 min a mixture of 0.5 ml of enzyme source solution and an equal volume of a 6-mM lactose solution in 0.1 M sodium phosphate buffer at the appropriate pH (that is, pH 7.0 for the intracellular or pH 5.0 for the extracellular samples). Lactose hydrolysis was stopped by heat inactivation at 80 °C for 5 min. Control reactions without added lactose were measured to quantify any residual lactose/glucose in each cell-free extract/culture medium sample. Phosphate buffer served as a blank reference for the reactions at either pH. The amount of D-glucose liberated from lactose by enzymatic hydrolysis as described above, was determined using the D-glucose oxidase-peroxidase method.<sup>41</sup> The D-glucose assay kit (Sigma-Aldrich) was used following the manufacturer's instructions. One unit (U) of lactose hydrolase activity corresponds to the formation of 1  $\mu$ mol of glucose per minute, and specific activity was expressed as U mg<sup>-1</sup> protein.

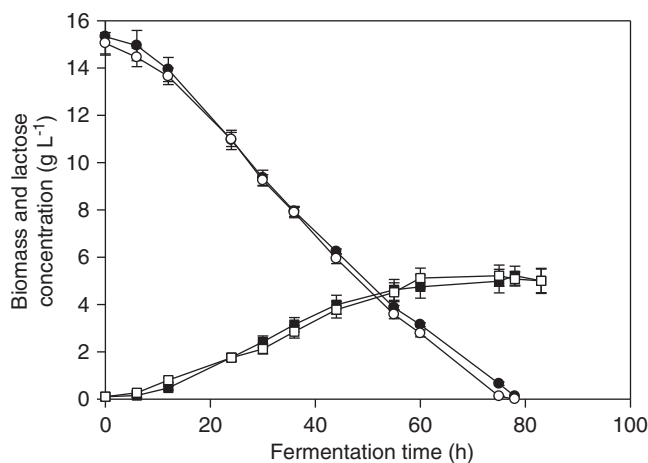
### Reproducibility

All the analytical and biochemical data presented are the means of three to seven independent experiments. Data were analyzed and visualized with SigmaPlot software (Jandel Scientific, San Rafael, CA, USA), and for each procedure, s.d. were determined. In the case of induction experiments using pregrown, transferred biomass, the significance of changes in enzyme activity relative to the non-induced, non-repressed growth condition (glycerol) was assessed using Student's *t*-test with *P*-values given in the Results section.

## RESULTS

### Growth of *P. chrysogenum* on lactose

To gain a first insight, both the wild-type reference strain as well as the industrially improved progeny strain AS-P-78 were grown on lactose as the sole carbon source. Both strains showed remarkably similar growth under batch conditions, consuming 15 g l<sup>-1</sup> lactose in about 74 h (Figure 1) and achieving a maximal specific growth rate of  $\mu = 0.045\text{--}0.047\text{ h}^{-1}$ , respectively, during the rapid growth phase. Differences in maximal biomass concentrations (5.2 g l<sup>-1</sup> and 5.3 g l<sup>-1</sup>, respectively) and the yield values ( $Y_{X/S} = 0.35\text{--}0.36$ ) achieved were not significant ( $P < 0.1\%$ ). It has to be noted that the presence of 0.01% peptone (that is, < 1% of the initial lactose concentration) in the (minimal) medium considerably shortened the lag period at the



**Figure 1** Cultivation of *P. chrysogenum* NRRL 1951 (empty symbols) and *P. chrysogenum* AS-P-78 (filled symbols) on lactose. Time profile of biomass production (squares) and residual lactose concentration (circles).

onset of growth from ~20 h to ~4 h for both the wild-type as well as the As-P-78 strains without essentially affecting the performance (growth rate) in the rapid growth phase. We presume that some undefined component(s) of peptone stimulate(s) spore germination, effectively resulting in an increased level of synchronization of the culture. Similar effects have been described in *A. nidulans*<sup>42</sup> and *T. reesei*.<sup>43</sup>

Data from these batch fermentations strongly suggested that the structural genetic elements required for lactose assimilation are present in the *P. chrysogenum* genome and that they are expressed and translated into enzymes in the germlings upon induction.

#### bGal activity in *P. chrysogenum*: artificial vs natural substrates

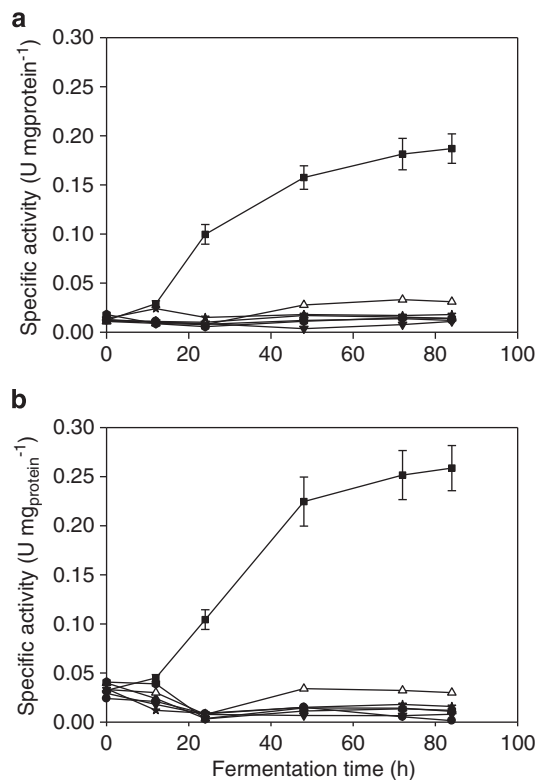
Lactose is an unnatural carbon source for fungi, while both extra- and intracellular bGal activities—for practical reasons—are traditionally defined as ONPG hydrolases. To test whether enzyme activity data obtained with the artificial substrate correspond to the ones obtained with lactose, we compared the extra- and intracellular bGal activity of both *P. chrysogenum* strains formed on the carbon sources that were used during the induction studies (see later) at two different concentrations (for example, 1 and 25 mM). As Supplementary Table S1 shows, while ONPG as an *in vitro* substrate indeed yielded slightly higher bGal activity values, the differences never exceeded 15% of the lactose-hydrolysis values ( $P < 0.1\%$ ). Essentially identical data were obtained at 25 and 1 mM inducer concentrations ( $P < 0.1\%$ ). We therefore considered the ONPG-based assay appropriate for the purpose of this study in *P. chrysogenum*.

#### Time profiles of extra- and intracellular bGal activity in *P. chrysogenum*

The time course of bGal activity formation on lactose as a sole carbon source in batch fermentations were analyzed using transferred mycelia pregrown on glycerol as inoculum. In both strains, the time profile of sugar depletion concurred with the presence of both intra- and extracellular bGal activities (Figures 1–3), measured as ONPG hydrolases. Activity on lactose rapidly increased during the phase of fast growth and rapid carbon substrate consumption and subsequently stabilized (Figures 2 and 3). Interestingly, in contrast to the intracellular bGal activity in *A. nidulans*,<sup>44</sup> both the extra- and the intracellular bGal activities of cultures growing on D-galactose were low, hardly above the background. On the other hand, a moderate extracellular (but not intracellular) ONPG-hydrolysing activity could be measured on L-arabinose as a sole carbon source (Figures 2 and 3), reaching a maximal value of approximately half of the one measured in lactose media in both of the two investigated strains. Upon growth on other commonly occurring monosaccharides, such as D-glucose, D-fructose, as well as on glycerol, neither extra- nor intracellular bGal could be detected in any of the strains.

#### Induction of $\beta$ -galactosidase activity of *P. chrysogenum*

In general, the use of alternative (or poorer) carbon sources (that is, other than glucose) requires induction of the catabolic pathway of the growth substrate in question, and induction indeed accounts for a major part of the bGal activity formed by fungi.<sup>45</sup> To investigate bGal induction in *P. chrysogenum*, mycelia from both strains were pregrown on glycerol—a ‘neutral’, that is, neither an inducing nor a repressing carbon source—and then transferred to a variety of sole carbon sources at different concentrations (see Materials and Methods for details). As Supplementary Table S1 shows, intracellular bGal activity appeared only on lactose, whereas its two monomers (D-glucose and D-galactose), the pentoses (D-xylose and L-arabinose)



**Figure 2** Time profile of the specific intracellular  $\beta$ -galactosidase activity of *P. chrysogenum* NRRL 1951 (panel a) and *P. chrysogenum* AS-P-78 (panel b) grown on D-glucose (●), D-fructose (▲), lactose (■), D-galactose (Δ), glycerol (▼), D-xylose (□) and L-arabinose (○) as a sole carbon source. For the clarity of the plots, error bars are shown for the lactose-grown samples only.

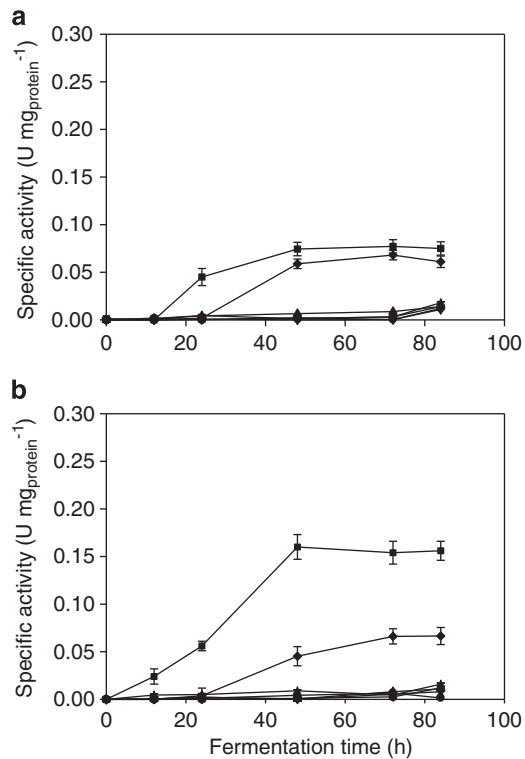
as well as D-fructose and glycerol were unable to induce bGal activity (nota bene (NB): non-significant D-galactose-induced levels ( $P < 0.1\%$ ) were detected in the NRRL 1951 strain). As substrate concentrations in the growth medium decreased during the course of the experiments, this result is apparently not due to inducer exclusion.

Analysis of the extracellular bGal activity formation vs carbon source revealed only one notable difference to the intracellular induction profile: L-arabinose appeared to affect bGal activity ( $P < 0.1\%$ ), reaching a level of ~65% of that attained on lactose (Supplementary Table S1). None of the other monosaccharide sugars tested induced any detectable bGal activity in the wild-type or the penicillin producer strain of *P. chrysogenum*. Activity profiles were qualitatively similar, but both activities—the extracellular in particular—appeared to be higher in strain AS-P-78 in the later stages of cultivation (for example, after 48 h). However, the extra enzyme activity did not stimulate growth performance or lactose consumption by AS-P-78 (Figure 1), which could suggest that sugar uptake may be the rate-limiting step of lactose catabolism.

#### *In silico* selection of glycosyl hydrolase and permease genes potentially involved in lactose assimilation

Among the *Aspergilli*—the sister genus of *Penicillium* within the Eurotiales order of the Eurotiomycetes<sup>46</sup>—both extra- and intracellular lactose hydrolysis modes have been described. *A. niger* hydrolyzes lactose extracellularly by means of (at least one) lactose-inducible, family 35 glycosyl hydrolase (GH35) called LacA,<sup>12,47</sup> corresponding to the annotated locus An01g12150 in the *A. niger*





**Figure 3** Time profile of the specific extracellular  $\beta$ -galactosidase activity of *P. chrysogenum* NRRL 1951 (panel a) and *P. chrysogenum* AS-P-78 (panel b) grown on D-glucose (●), D-fructose (▲), lactose (■), D-galactose (Δ), glycerol (▼), D-xylose (□) and L-arabinose (◆) as sole carbon source. For the clarity of the plots, error bars are shown uniquely for the lactose- and L-arabinose-grown samples.

CBS 513.88 genome sequences.<sup>48</sup> The CBS 513.88 genome further specifies four *lacA*-paralog genes at loci An14g05820, An01g10350, An06g00290 and An07g04420. The five *A. niger* GH35 proteins phylogenetically correspond to four clearly defined clades with common origin, representing four paralog genes found in various classes of *Peizizomycotina* (Supplementary Figure S1). The *P. chrysogenum* Wisconsin 54–1225 genome harbors three GH35 paralog genes at annotated loci Pc16g12750, Pc06g00600 and Pc14g01510, all of them specifying a predicted signal sequence for secretion, which were selected for transcript analysis to assess possible implication in lactose assimilation (see below), and were named *bgaA*, *bgaB* and *bgaC*, respectively (Table 1). The *lacA*-ortholog at locus Pc16g12750 (for example, *bgaA*) corresponds to the sequenced bGal genes from closely related *Penicillium* species *P. expansum* and *P. canescens* (NB, accession numbers ACD75821, CAA49852 and CAF32457). A full-length ortholog for An14g05820 (clade 2 in Supplementary Figure S1) could not be found in the *P. chrysogenum* Wisconsin 54–1225 genome nor in that of *P. chrysogenum sensu strictu*, as the encoding gene—intact in *P. paxilli* and *P. digitatum*—has apparently receded to a pseudogene (genome contig Pc00c21, nucleotide coordinates 2240190 to 2243618 on the opposite strand).

On the other hand, *A. nidulans* hydrolyzes lactose intracellularly. Recently, we identified a gene cluster consisting of an intracellular bGal gene (*bgaD*), encoding a GH2 enzyme with pronounced activity against artificial bGal substrates, and a lactose permease (*lacpA*) gene, that coordinately respond to lactose induction.<sup>11</sup> This gene cluster (loci AN3199 and AN3201) is conserved in a considerable number of

ascomycetes. BgaD orthologs could be found in both *P. chrysogenum* as well as in *P. paxilli*, whereas *P. decumbens* specifies a paralog GH2 protein, clustering together with *A. nidulans* AN2463 and GH2 proteins from the two sequenced *Talaromyces*, distally related species of Eurotiales (Supplementary Figure S2). The sole AN3201-ortholog GH2 gene in *P. chrysogenum* (locus Pc22g14540—TPA accession BK008499; see Figure 1 in Fekete *et al.*<sup>11</sup>) was selected to probe the possible involvement in intracellular lactose hydrolysis in NRRL 1951 and AS-P-78, and was named *bgaD* (Table 1).

The *bgaD-lacpA* gene cluster in *A. nidulans* is interrupted by another gene predicted to encode an intracellular GH2 enzyme (accession JQ681216),<sup>11</sup> (see Supplementary Figure S3 associated with that paper). This gene (at locus AN3200) does not respond to lactose induction but is expressed at a low, constitutive level. The Wisconsin 54–1255 ortholog of this GH2 gene (at locus Pc12g11750, named *bgaE* in Table 1) can be found in a *Penicillium*-specific branch in a phylogenetic tree of AN3200 orthologs (Supplementary Figure S3), and was also selected for expression analysis in NRRL 1951 and AS-P-78.

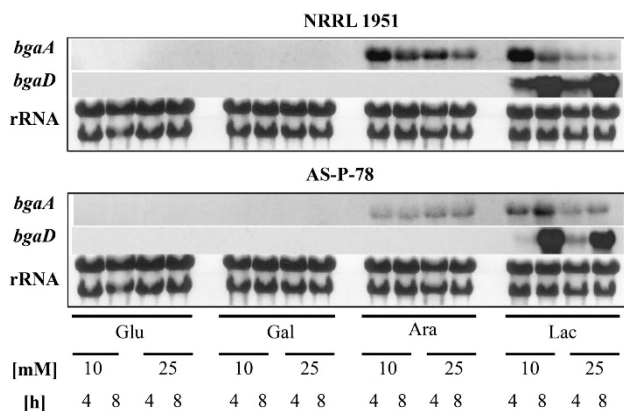
In *P. chrysogenum*, the sugar porter gene (locus Pc22g14530) transcribed divergently from *bgaD* is not closely related to the characterized *A. nidulans* lactose permease gene (see Figure 2 in Fekete *et al.*<sup>11</sup>). A screen of the genome sequences strongly suggested that *P. chrysogenum* Wisconsin 54–1255 as well as *P. chrysogenum sensu strictu* lack a *LacpA* ortholog. However, there is >1 physiological lactose uptake system operative in *A. nidulans*<sup>11</sup> and its genome specifies three sugar porter genes (at loci AN1577, AN6831 and AN2814) that could be considered structural orthologs of the paralogs of *lacpA*, which encodes the lactose transporter of higher affinity (see Supplementary Figure S5 in Fekete *et al.*<sup>11</sup>). The Wisconsin 54–1255 genome harbors orthologs for two of these three *A. nidulans lacpA*-paralog genes, at annotated loci Pc13g08630 and Pc16g06850, while the same is true for *P. chrysogenum sensu strictu*. (Supplementary Figure S4) The sugar porter genes (named *lacA* and *lacB*, see Table 1) at the two mentioned Wisconsin 54–1255 loci were selected to monitor their expression at the transcript level.

#### Expression profiling of the putative bGal and lactose permease genes

Specific bGal activity data on a variety of carbon sources showed that no enzyme was formed on D-glucose, D-xylose, glycerol and D-fructose. Therefore, transcript analysis of the genes identified *in silico* as being putatively involved in lactose catabolism (Table 1) was performed on lactose, D-galactose and L-arabinose, using D-glucose as a negative reference. Pregrown mycelia were treated similarly as described at the induction experiments, except that samples were taken at two different time points, that is, at 4 and 8 h after the transfer of mycelia.

Out of the five *P. chrysogenum* candidate genes identified as putatively encoding a bGal enzyme, two of the three extracellular genes (*bgaB* and *bgaC*) were not expressed on lactose at any time point or concentration used, in any of the two strains (data not shown). Expression characteristics of the remaining three hydrolase genes (the extracellular *bgaA* as well as the intracellular *bgaD* and *bgaE*) was identical in the two *P. chrysogenum* strains and was apparently not influenced by the lactose concentration used (for example, 10 or 25 mM). Remarkably, elongated incubation time (up to 8 h) relative to the 4-h-long period clearly increased the transcript abundance of *bgaD*.

In the closely related fungus *A. nidulans*, the *bgaD*-ortholog of *P. chrysogenum* (also called *bgaD*) is solely responsible for intracellular



**Figure 4** Transcript analysis of the induction spectrum of two putative  $\beta$ -galactosidase genes (*bgaA*, putatively encoding an extracellular- and *bgaD*, putatively encoding an intracellular bGal) in response to D-glucose (Glu), D-galactose (Gal), L-arabinose (Ara) and lactose (Lac) in *P. chrysogenum* NRRL 1951 and *P. chrysogenum* AS-P-78.

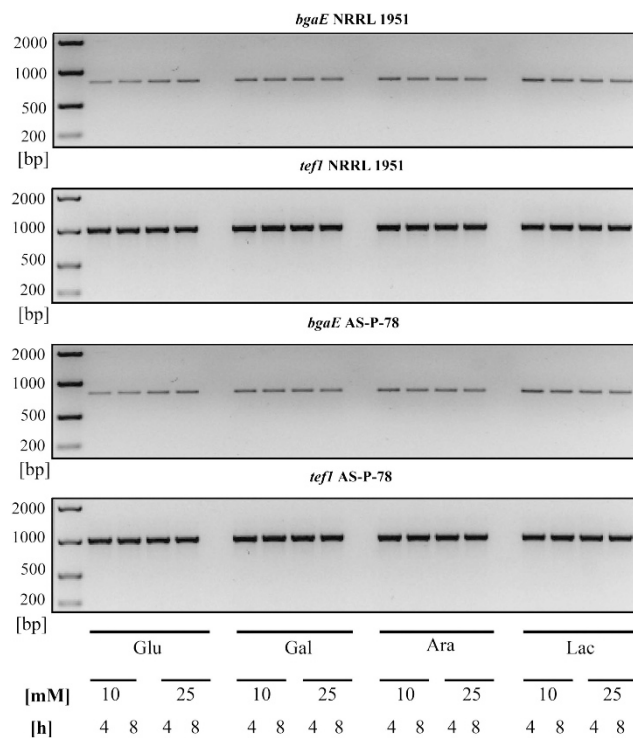
5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside hydrolase formation.<sup>11</sup> As *P. chrysogenum* did not form intracellular bGal activity on any carbon source but lactose, it was no surprise that transcription of *bgaD* exclusively occurred on lactose, and could not be provoked by other carbon sources including D-galactose, one of the products of lactose hydrolysis (Figures 4 and 5). On the other hand, similarly to its *A. nidulans*-ortholog AN3200, *bgaE* exhibited a weak, constitutive expression under all conditions tested. *bgaE* transcripts could hardly be detected by Northern analysis, and we used semiquantitative RT-PCR to probe the expression of this particular gene. *bgaE* was expressed at a low basal level under all tested conditions, including those in which we were unable to measure either ONPG or lactose hydrolysis. Similarly to its ortholog in *A. nidulans*, *bgaE* unlikely has an important role in lactose catabolism in *P. chrysogenum*.

As for the two putative lactose permease genes, *lacA* was expressed exclusively on lactose in both *P. chrysogenum* strains at both inducer concentrations and incubation times (Figure 6). Interestingly, *lacA* expression levels appeared to be considerably higher at 4 h after the medium shift than at 8 h, that is, exactly the reverse of what has been observed for the intracellular *bgaD* gene. On the other hand, *lacB* transcript could not be observed on lactose (Figure 6) or on any other carbon sources tested (data not shown), indicating its irrelevance in the assimilation of the disaccharide.

## DISCUSSION

Lactose is the major carbohydrate of milk. Although essential for newborn and young mammals,<sup>2</sup> it is unavailable in the majority of habitats colonized by microorganisms. Therefore, unsurprisingly, many bacterial, yeast and fungal species lack the ability to utilize lactose, and those that can often exhibit low assimilation rates. Regulation of lactose utilization by means of the *lac* operon in *Escherichia coli* is a classic paradigm in prokaryotic genetics,<sup>49</sup> and the *LAC* regulon of the yeast *K. lactis* is a model system for transcriptional control in lower eukaryotes.<sup>50</sup> However, several aspects of lactose utilization in filamentous fungi, including the industrial cell factory *P. chrysogenum* is poorly understood.

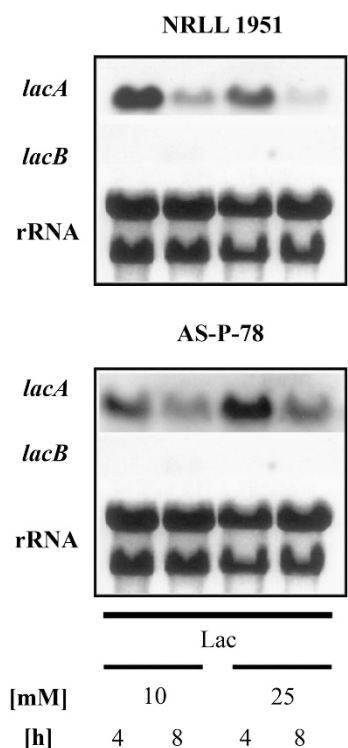
A recent taxonomic reappraisal of the *Trichocomaceae* family (*Ascomycota*; *Peizozymycotina*; *Eurotiomycetes*; *Eurotiales*) established *Penicillium* (*sensu strictu*) and *Aspergillus* (*sensu strictu*) as sister genera within the new *Aspergillaceae* family.<sup>46</sup> Various members of the



**Figure 5** Expression characteristics of the *bgaE* gene, putatively encoding an intracellular  $\beta$ -galactosidase in *P. chrysogenum* NRRL 1951 and *P. chrysogenum* AS-P-78. Symbols used are identical to Figure 4. Expression of the gene encoding the eukaryotic translation elongation factor 1-alpha component (*tef1*) served as a constitutive control for the reverse transcriptase-PCR analysis.

*Aspergillus* subgenera *Circumdati*, *Nidulanti*, *Negri* and *Fumigati* are therefore the organisms most closely related to *P. chrysogenum* (*sensu lato*) for which whole-genome sequences and annotation are available. With few exceptions,<sup>51,52</sup> fungi tend to employ only one of the two principal strategies—extracellular or intracellular hydrolysis—for the catabolism of lactose, typified by *A. niger* and *A. nidulans*, respectively. Here, we report evidence that indicate that *P. chrysogenum* (*sensu lato*) features a dual lactose assimilation scheme that comprises an extracellular bGal, a (putative) lactose permease and an intracellular bGals. *In silico* analysis revealed that the *P. chrysogenum* genome features another two putative extracellular bGal-encoding GH35 genes at the annotated loci Pc14g01510 and Pc06g00600 (*bgaB* and *bgaC*), but these genes were not expressed under the conditions tested and are therefore unlikely to be physiologically relevant for growth on lactose. Nevertheless, production of bGals isozymes by one species would not be without precedent among the fungi: *A. carbonarius* produces two extracellular bGal with differences in their amino-acid compositions,<sup>53</sup> whereas the acidophilic fungus *Teratosphaeria acidotherma* (*Ascomycota*; *Peizozymycotina*; *Dothideomycetes*; *Capnodiales*) was described as producing four different intracellular bGals, each with a different amino-acid composition.<sup>54</sup>

Growth characteristics (for example, maximal specific growth rate, biomass yield vs carbon consumed) of *P. chrysogenum* on lactose were remarkably similar to *A. nidulans* or *T. reesei*. Interesting differences were found, however, in the regulation of bGal transcript/activity formation vs the carbon source available. Most notably, neither the extra- nor the intracellular bGal activity, and none of the putative *Bgal* and permease genes could be induced by D-galactose, whereas in



**Figure 6** Transcript analysis of the induction spectrum of the two putative lactose permease genes (*lacA* and *lacB*) in response to lactose (Lac) in *P. chrysogenum* NRRL 1951 and *P. chrysogenum* AS-P-78. No transcript was observed for any of the other carbon sources tested. As a control for the quality and quantity of the RNA, ribosomal RNA (28S and 18S) was visualized with ethidium bromide; the negative of the original image is shown at the bottom (rRNA).

all fungi (and indeed, bacteria<sup>55</sup>) studied to date, D-galactose was at least as good an inducer of the Bgal-encoding genes as lactose.<sup>43,44,56</sup> As the monosaccharide D-galactose is considered a repressing carbon source<sup>45</sup> and fungal bGal genes are typically subject to general carbon catabolite repression,<sup>11,22,43,57</sup> we hypothesized that any inducing effect of D-galactose in *P. chrysogenum* might be counteracted by a simultaneous feedback carbon catabolite repression. To test this hypothesis, we decreased inducer concentrations from the standard 25 and 10 to 1 mM and even down to 0.1 mM. Irrespective of the repressive nature of a given carbon source, applying such low concentrations to medium-shifted biomass will result in a low specific growth rate and subsequently, carbon derepression.<sup>58</sup> However, none of the putative bGal genes were found to be overexpressed even at the lowest (for example, 0.1 mM) of the D-galactose concentrations tested, either at 1, 2 or 4 h after the transfer of mycelia (data not shown), suggesting that in *P. chrysogenum*, these genes do not respond to the monosaccharide that is one of the primary products of lactose hydrolysis.

An intriguing trait in the regulation of filamentous fungal bGal-encoding genes is their apparent inducibility by pentose monomers that derive from hemicellulose degradation, such as L-arabinose or D-xylose.<sup>25,56</sup> The reason is unknown but the phenomenon could be related to their occurrence in natural polysaccharides that also contain galactose, which serve as carbon sources for saprophytic and phytopathogenic fungi, such as, for example, arabinogalactan. However, from our experiments with *P. chrysogenum*, it was evident that none

of its predicted bGal genes responded to D-xylose, nor could we measure any ONPG hydrolase activity. In contrast, L-arabinose appeared to promote moderate expression of, uniquely, the *bgaA* gene, whose product is likely responsible for the extracellular ONPG-hydrolase (and lactase) activity measured in L-arabinose cultures, like has been observed previously for the ortholog bGal in the related fungus *P. canescens*.<sup>25</sup> Concomitant with the observation that only the extracellular bGal is expressed in the presence of L-arabinose, the *P. chrysogenum* Wisconsin 54–1255 genome sequences do not specify an ortholog of the intracellular L-arabinose-responsive BgaD-paralog GH2 of *A. nidulans* (locus AN2364). In *A. nidulans*, L-arabinose was shown to modestly affect the expression of *bgaD* (intracellular bGal) and a paralog GH2 gene at locus AN2463,<sup>11</sup> whereas the latter gene does not respond to lactose. It was also shown that the apparent inductive effect of L-arabinose on *bgaD* was not additive to derepression in *creA* loss-of-function mutants, in contrast to the situation with lactose. This could suggest that L-arabinose is derepressing rather than inducing *A. nidulans bgaD* expression. Unfortunately, publicly available *creA*-mutants do not exist in *P. chrysogenum*, so we were not able to test whether L-arabinose derepresses rather than induces the expression of the extracellular bGal encoded by *bgaA*.

On the other hand, it is well known that certain bGals hydrolyze alpha-L-arabinopyranoside substrates *in vitro*, reflecting the similarity between the beta-D-galactopyranose and alpha-L-arabinopyranose hemiacetal configurations.<sup>59</sup> Consequently, it could be that *P. chrysogenum* produces (an) extracellular alpha-L-arabinopyranosidase(s) that exhibit(s) side activity against ONPG. However, extracellular hydrolyase activity apparently induced by L-arabinose in *P. chrysogenum* split both ONPG and lactose as the *in vitro* substrate, making the alpha-L-arabinopyranosidase hypothesis less likely.

ONPG enzyme activities correlated with transcript formation patterns of *bgaA* as well as of *bgaD*, thus the observed effects on enzyme activity profiles may well reflect transcriptional regulation. From these results, one may conclude that the structural components of intracellular lactose hydrolysis in *P. chrysogenum* resemble those in *A. nidulans* with a major, inducible (*bgaD*) bGal gene, an inducible permease (*lacA*) and possibly, a minor, constitutive GH2 gene (*bgaE*). Meanwhile, the means for extracellular lactose hydrolysis bear similarity to those in *A. niger*.<sup>56</sup> This strongly suggests that *bgaA* and *bgaD* encode the (major) hydrolases involved in extra- and intracellular lactose breakdown, respectively, in *P. chrysogenum*.

In *A. nidulans*, the deletion of the lactose permease, *LacpA*, did not result in the inability to take lactose from the medium and therefore a second uptake system is operative that enables growth on lactose.<sup>11</sup> The *P. chrysogenum* Wisconsin 54–1255 genome does not seem to specify a *lacpA* ortholog gene (see Supplementary Figure S4). The fungal bGal/lactose permease gene cluster seems to have a complex evolutionary history in ascomycetes with duplication events and adaptive loss of genes.

In the case of the sequenced *Penicillium* species, the ortholog of *BgaD* appears to lack from *P. decumbens* and *P. digitatum*. On the other hand, the *P. paxilli* genome specifies two BgaD-like proteins, one of which clusters with orthologs from *Aspergilli* in a phylogenetic subclade that also contains proteins from two species of the Chaetothyriales class of the Eurotiomycetes, *Cladophialophora carriarii* and *Exophiala dermatitidis*. The second *P. paxilli* BgaD protein clusters with the solitary orthologs found in *P. chrysogenum sensu strictu* and *P. chrysogenum* Wisconsin 54–1225 in a clade that primarily contains Sordariomycetes proteins. In both *P. chrysogenum* genomes, the sugar porter gene transcribed divergently from *bgaD* is



not closely related to the characterized *A. nidulans* lactose permease gene, and the same is true for the *P. paxilli* *bgaD*-ortholog GH2 gene in the 'Sordariomycetes' clade (see Supplementary Figure S2). We could confirm that the *MFS* gene at locus Pc22g14530 is not responding to lactose and not co-expressed with *bgaD* (results not shown). Conversely, the second *P. paxilli* gene for the GH2 that clusters with *A. nidulans* BgaD and the orthologs from other *Aspergilli*, does have the *lacpA* permease gene divergently transcribed from it. Our phylogenetic analyses thus suggest that the current *P. chrysogenum* ortholog BgaD protein arises from a horizontal gene transfer (originating from an unknown Sordariomycete) and that after this acquisition, the original 'Eurotiomycetes' ortholog—its original intracellular bGal gene—was lost along with the *lacpA* ortholog lactose transporter gene clustered with it. These events may also explain the notable differences in *bgaD* expression responses in *A. nidulans* and *P. chrysogenum*, as apparent induction of the intracellular bGal by galactose and L-arabinose only occur in the former.

*P. chrysogenum* seems to harbor only one physiologically relevant lactose transporter, LacA—a structural paralog of *A. nidulans* LacpA and the ortholog of the *A. nidulans* permease encoded at locus AN2814. LacA is present in all screened *Penicillium* genomes including in *P. digitatum*, a species that lacks the intracellular GH2 gene structurally related to *bgaD* (for which two strains have been sequenced, see Materials and Methods section). Interestingly, the ortholog in *N. crassa* (locus NCU00801) is described as a transporter of cellobiose (1,4-*O*-β-D-glucopyranosyl-D-glucose), a disaccharide that is very similar to lactose (1,4-*O*-β-D-galactopyranosyl-D-glucose).<sup>60</sup> The other gene we identified as a putative lactose transporter, LacB—orthologs of which occur only in *P. chrysogenum* Wisconsin 54–1255, *P. chrysogenum sensu strictu* and *P. digitatum* and are missing from *P. paxilli* and *P. decumbrens*—was not found to be expressed on any of the carbon sources tested. Interestingly, in the cellulase enzyme producer *T. reesei*, an ortholog of *P. chrysogenum* LacB and *A. nidulans* AN6831 (a third paralog of LacpA<sup>11</sup>) identified as protein ID Trire2:3405 appears to be essential for growth on lactose,<sup>61</sup> as deletion of the encoding gene completely blocked the ability of *T. reesei* to grow on lactose, without affecting growth on D-glucose or D-galactose. This recent finding contradicts the long-standing assumption<sup>62,63</sup> that lactose catabolism in *T. reesei* is exclusively extracellular, as the extracellular GH35 *Bga1* is normally expressed by this transporter deletant. The intracellular lactose-hydrolysing enzyme is yet to be identified, however.<sup>61,64</sup>

It is well documented that increased penicillin production in the early progeny of various industrial improvement programs, including AS-P-78, coincides with genic amplification of the penicillin biosynthesis gene cluster,<sup>29</sup> but it was also recognized that the observed overexpression of penicillin biosynthesis genes could not be explained solely by proliferation of the cluster amplicon.<sup>30</sup> It was suggested that part of the improved titers are the consequences of mutations *in trans* resulting in altered cluster induction and/or affecting the general regulatory circuits ruling primary metabolism to which the penicillin cluster is subject.<sup>65</sup> Lactose is a gratuitous carbon source for filamentous fungi that yields slow growth and associated carbon derepression.<sup>11,66</sup> However, a comparative analysis of growth parameters performed here clearly showed that the increased penicillin-producing potential of AS-P-78 cannot be related to any reduction in the assimilation rate of lactose, relative to the wild-type reference strain NRRL 1951. Therefore, data available at this stage suggest that lactose metabolism of *P. chrysogenum* lacks correlation to the penicillin-producing potential of the fungus, at least, in the pedigree of the early generation, improved titer strain AS-P-78.

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