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Identification of a permease gene involved in lactose utilisation in *Aspergillus nidulans* ☆

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ABSTRACT

Lactose is intracellularly hydrolysed by *Aspergillus nidulans*. Classical mutation mapping data and the physical characteristics of the previously purified glycosyl hydrolase facilitated identification of the clustered, divergently transcribed intracellular β-galactosidase (*bgaD*) and lactose permease (*lacpA*) genes. At the transcript level, *bgaD* and *lacpA* were coordinately expressed in response to D-galactose, lactose or L-arabinose, while no transcription was detectable in the additional presence of glucose. In contrast, *creA* loss-of-function mutants derepressed for both genes to a considerable extent (even) under non-inducing or repressing growth conditions. Lactose- and D-galactose induction nevertheless occurred only in the absence of glucose, indicating a regulatory role for CreA-independent repression. Remarkably, *bgaD* deletion mutants grew normal on lactose. In contrast, *lacpA* deletants grew at a much slower rate in lactose liquid medium than wild-type while strains that carried more than one copy of *lacpA* grew faster, showing that transport is the limiting step in lactose catabolism. The effect of *lacpA* gene deletion on lactose uptake was exacerbated at lower substrate concentrations, evidence for the existence of a second transport system with a lower affinity for this disaccharide in *A. nidulans*.

1. Introduction

Microbial β-D-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) is an important enzyme in the food- and fermentation industry due to its ability to hydrolyse lactose (milk sugar; β-D-galactopyranosyl-(1,4)-D-glucopyranose). Its application in food production and the technological challenges in its use have been reviewed by, e.g., Panesar et al. (2006) and Rubio-Texeira (2006). Fungal β-galactosidases include extracellular enzymes, characterised by an acidic pH optimum, and intracellular ones, that typically function optimally at neutral pH. The review literature often gives the impression that only lactose-assimilating yeasts are capable of synthesising the pH-neutral enzymes. However, *Neurospora crassa*, for instance, simultaneously produces both isozymes on lactose media (Bates and Woodward, 1964). In *Aspergillus nidulans*, only a lactose- and D-galactose inducible intracellular activity with a neutral pH optimum has been described (Paszewski et al., 1970; Fantes and Roberts, 1973; Díaz et al., 1996; Fekete et al., 2002). The native enzyme, assayed with the artificial substrate *ortho*-nitrophenyl-β-D-galactopyranoside (ONP-Gal), has an estimated molecular mass of 450 kDal and appears to be a homotetramer of ~120-kDal subunits.

In biotech industry, whey – the abundant lactose-rich by-product of cheese manufacture – has long been used as a cheap growth substrate for the production of valuable fungal metabolites, like penicillin by *Penicillium chrysogenum* (Brakhage, 1998) or cellulase and heterologous proteins by *Trichoderma reesei* (Persson et al., 1991). Lactose is a poor carbon source for filamentous fungi and this facilitates the production of secondary metabolites and polymer-degrading enzymes, biosyntheses of which are generally under strong carbon catabolite repression. This carbon-derepressing effect of lactose has been shown to operate at the level of transcription in *A. nidulans* (Flipphi et al., 2003a) and it can be mimicked in fermentations by feeding glucose at increasingly lower dilution rates (Ilyés et al., 2004), suggesting that carbon catabolite repression in this model organism is dependent on growth rate. In fungi

Abbreviations: DCW, dry cellular weight; d.p.m, disintegrations per minute; GH2, family 2 glycosyl hydrolase; GH35, family 35 glycosyl hydrolase; MFS, major facilitator superfamily (protein); ONP-Gal, *ortho*-nitrophenyl-β-D-galactopyranoside; RT-PCR, reverse transcriptase-polymerase chain reaction; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

☆ This paper is dedicated to Prof. Attila Szentirmai, founder of the Industrial Biotechnology School at the University of Debrecen, at the occasion of his eighty second birthday.

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that hydrolyse lactose intracellularly, such as *A. nidulans*, the rate-limiting event might be the uptake of the disaccharide. Our aim was, therefore, to identify the functional lactose permease gene in *A. nidulans* and to characterise lactose uptake with the help of gene-deleted strains.

A. nidulans mutants in lactose utilisation can be distinguished in uptake mutants, that still produce ONP-Gal hydrolase in response to D-galactose induction, and β-galactosidase-negative mutants. Classical mutants that were defective for both uptake and hydrolysis of ONP-Gal have never been described. Gajewski et al. (1972) selected a number of mutations, the large majority of which mapped to two loci on linkage group (chromosome) VI, one for lactose uptake (*lacA*) and the other for β-galactosidase (*lacG*). Although the authors reported difficulties in crossing their mutant strains, recombination frequencies between *lacA* and *lacG* mutations ranged from 12% to 19%, suggesting that the two loci are genetically linked. The leaky ''β-galactosidase'' mutation *lacC24*, the only one not mapping on chromosome (Chr.) VI, was later shown to be a combination of two proline catabolism mutations completely unrelated to lactose catabolism (Jones et al., 1981). Somewhat surprisingly, Fantes and Roberts (1973) described a group of mutants that could not produce ONP-Gal hydrolysing activity but grew normally on lactose plates. These mutations mapped to three independent loci (*bgaA–C*). One of the *bgaA* mutations was thermosensitive and this characteristic prompted these authors to localise the structural gene for β-galactosidase as on Chr. III.

Although a membrane protein can be readily identified on the basis of its amino acid sequence as a member of the Major Facilitator Superfamily (MFS) (cf. Pao et al., 1998), further *in silico* characterisation vis-á-vis a prediction of the transported substrate(s) can be difficult due to an apparent lack of conserved signature sequences beyond the similarity intrinsic to the twelve transmembranal domains. The genome of the soil-borne saprophyte *A. nidulans* is predicted to harbour around 400 genes encoding MFS proteins, more than 100 of which were assigned to the Sugar Porter Family (Wortman et al., 2009). The sugar transport potential of *A. nidulans* can be appreciated when compared to the predicted sugar porter complement in saccharomycetes such as *Candida albicans* (20 sugar porters), *Kluyveromyces lactis* (20) or *Yarrowia lipolytica* (23) (Palma et al., 2007). However, in the case of lactose transport in *A. nidulans* it might be possible to identify, *in silico*, a single candidate gene, using existing genetic and biochemical data in relation to the re-annotated genome sequences. Our strategy was first try to identify a structural gene for β-galactosidase from the genome sequences and, if such a gene were to be located on Chr. VI, screen for sugar porter genes in the vicinity that might correspond to the *lacA* lactose uptake locus.

2. Materials and methods

2.1. A. nidulans strains, media, and culture conditions

A. nidulans strains and transformants used in this study are listed in Supplementary Table 1. Minimal media for shake flask cultivations and fermentations were formulated and inoculated as described by Fekete et al. (2002). Vitamins and other supplements were added from sterile stock solutions. Carbon sources were used at 1 % (w/v) end concentration unless stated differently. Batch cultures were incubated at 37 °C in Erlenmeyer flasks in a rotary shaker at 200 strokes per minute. All chemicals (Sigma–Aldrich) were of analytical grade.

2.2. Classical genetic techniques and transformation

Conventional genetic techniques (Clutterbuck, 1974) were employed to exchange extant markers by meiotic recombination. β-Galactosidase was tested on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) at 100 μg/ml end concentration. The ONP-Gal hydrolase-negative mutation *bgaA0* (i.e., zero) (Fantes and Roberts, 1973) was recuperated from WG355, a strain in use as a transformable host for fungal promoter studies (e.g., Brakhage et al., 1992). The L-arginine-auxotrophy was crossed out using V088 to yield, a.o., strain V204. The *bgaA0* marker was verified on X-Gal plates.

A. nidulans transformations were performed basically as described by Tilburn et al. (1983), using Glucanex (Novozymes) as cell-wall lysing enzyme. Primary transformants were purified to single cell colonies and maintained on selective minimal plates.

2.3. 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 rapidly frozen in liquid nitrogen. For nucleic acid isolation, frozen biomass was ground to dry powder using liquid nitrogen-chilled mortar and pestle. Genomic DNA was extracted using Promega's Wizard SV Genomic DNA Purification System while total RNA was isolated with Promega's SV Total RNA Isolation System.

2.4. Northern and Southern blot analysis, and reverse transcription PCR (RT-PCR)

Standard procedures (Sambrook and Russell, 2001) were used for the quantification, denaturation, gel separation and nylon blotting of nucleic acids, and the hybridisation of the membranes. Agarose gels were charged with 5 μg DNA/RNA per slot. Probes were digoxigenin-labelled using the PCR DIG Probe Synthesis Kit (Roche Applied Science) primed with gene-specific oligonucleotides (Supplementary Table 2) off R21 genomic DNA. Hybridisation was visualised with Lumi-Film Chemiluminescent Detection film (Roche Applied Science).cDNA was synthesised from 1 μg of DNase I-treated total RNA using Oligo(dT) as a primer and Moloney murine leukemia virus reverse transcriptase (all provided by Fermentas). PCR was performed in a 25 μL volume containing 4 μL of cDNA, using AN3200-specific oligonucleotides as primers (Supplementary Table 2) and Taq polymerase (Promega). Cycling conditions after an initial denaturing at 95 °C for 2 min were: 40 cycles of 95 °C for 30 s, 59 °C for 1 min, and 72 °C for 45 s.

2.5. Generation of gene-deleted strains

A deletion cassette was constructed in vitro according to the double-joint PCR method (Yu et al., 2004). The cassette consisted of ~500–900 bp of the noncoding regions of the targetted gene (i.e., *bgaD* or *lacpA*) flanking the functional orotidine-5phosphate decarboxylase (*pyr4*) gene from *T. reesei* (2278 bp) (Gruber et al., 1990). Oligonucleotide primers that were used are listed in Supplementary Table 2. Protoplasts of *A. nidulans* uridine-auxotroph TN02A3 (Nayak et al., 2006) were transformed with 10 μg of either deletion cassette. Uridine-prototroph transformants were probed for the absence of *bgaD* or *lacpA* sequences by PCR, primed off genomic DNA using gene-specific primers. Selected gene-deleted strains were verified by Southern blot analysis.

2.6. Reintroduction of bgaD/lacpA in gene-deleted backgrounds

A characterised first generation deletant of either gene was crossed with strain M3091. Uridine-prototroph and riboflavin-auxotroph offspring was PCR-verified for the presence of the *nkuA* gene. Functional *bgaD* or *lacpA* genes were amplified off R21 genomic DNA using specific primers. 10 μg of the amplification product

was co-transformed with 1 μg of pTN2 (carrying the *riboB* gene from *Aspergillus fumigatus*; Nayak et al., 2006) into one of the *nkuA*-cured, second generation gene-deleted strains. Among the riboflavin-prototroph transformants, the presence of the re-introduced gene was probed by PCR and Southern blot analysis, and those that had re-acquired *bgaD* or *lacpA*, respectively, were phenotypically characterised.

2.7. Analytical methods

Mycelial dry weight was determined upon withdrawing two 5-ml aliquots from a culture, suction filtration of the biomass of each sample over a glass wool filter of known weight and drying the filter at 80 °C until constant weight. Figures were averaged and deviated maximally 13%. The concentration of D-glucose, D-galactose, L-arabinose, D-xylose, glycerol and lactose was determined by HPLC analysis with a proton exchange column (Bio-Rad Aminex HPX-87H) using isocratic elution with 10 mM H_2SO_4 at 55 °C and refractive index detection.

2.8. Lactose uptake experiments

Mycelia were grown for 18 h on lactose as the carbon source, harvested by gentle filtration over sterile cheese cloth, thoroughly washed with minimal medium without carbon source and resuspended in carbon-free minimal medium to a final density of 1 mg/ml. 10-ml aliquots were pre-incubated in 100-ml Erlenmeyers at 37 °C for 1 h in a shake waterbath at 100 strokes per minute. ^{14}C-radiolabelled lactose (Sigma: [D-glucose-1-14C] lactose, 60 mCi/mmol) was added to yield ~150,000 d.p.m (2500 Bq) per mg of biomass. Unlabelled lactose was added to give the lactose concentration desired.

The mycelial cultures were incubated for further 6 h at 37 °C under agitation while samples were withdrawn at regular intervals. Uptake of label was immediately stopped by pipetting a 1-ml mycelial sample into an Eppendorf tube containing 100 μl of a 1-M lactose solution and vigorously shaking the tube. Cellular debris was then spun down in an Eppendorf centrifuge at 10,000 g for 5 min. Residual d.p.m in the supernatant was determined using OptiPhase HiSafe3 scintillation fluid (Perkin-Elmer) in a Wallac liquid scintillation counter. For Table 1, the d.p.m figures were recalculated to μmoles of lactose. Uptake rate was expressed in μmoles per minute and gram of dry cellular weight (DCW).

2.9. Bio-informatics

For comparative analysis, gene-model corrected *A. nidulans* proteins were used as bait in TBLASTN screening (Altschul et al., 1997) of the NCBI nr and/or fungal databases. For phylogenetic analysis, protein sequences were aligned using CLUSTALW (Thompson et al., 1994). A phylogenetic tree was then constructed feeding the alignment into MEGA-4 software (Tamura et al., 2007) and running the neighbour-joining algorithm (Saitou and Nei, 1987) with default settings, unless stated otherwise. Stability of clades was evaluated by 1000 bootstrap rearrangements.

2.10. Structural analyses of bgaD and lacpA

The *bgaD* and *lacpA* genes were sequenced from overlapping PCR fragments amplified with specific oligonucleotides (Supplementary Table 2) off genomic DNA from various strains used in this study. The gene sequences from the various sources were identical. cDNA was also sequenced. The nucleotide sequences are available at GenBank under accession numbers FJ647189 (*lacpA*) and GU129977 (*bgaD*).

3. Results

3.1. In silico identification of an intracellular β-galactosidase gene in A. nidulans

Functionally characterised intracellular (pH-neutral) β-galactosidases belong to Glycosyl Hydrolase family 2 (GH2) (Cantarel et al., 2009). The genome annotation (Wortman et al., 2009) predicts nine genes for GH2 enzymes, on chromosomes Chr. I (one gene), Chr. V (one), Chr. VI (four) and Chr. VII (three genes). Not all of these will encode a β-galactosidase as filamentous fungal β-mannosidase (Takada et al., 1999), β-glucuronidase (Wenzl et al., 2005) and β-*exo*-glucosaminidase (Ike et al., 2006) are also classed as GH2. In addition, genes potentially encoding glycosyl hydrolases from Family 35 (GH35) (Cantarel et al., 2009) – typified by mammalian lactase (lactose galactohydrolase; EC 3.2.1.108) and *Aspergillus niger* extracellular β-galactosidase LacA (cf. Kumar et al., 1992) – were taken in consideration. The three putative GH35 genes are located on Chr. VI (one) and Chr. VIII (two intact genes and a pseudogene). The genome sequence thus dismisses the conclusion from Fantes and Roberts (1973) regarding the localisation of the structural β-galactosidase gene in *A. nidulans*.

From the five candidate loci for the β-galactosidase gene on Chr. VI, only one, AN3201, appeared to specify a gene big enough to produce a peptide of 120 kDal, the experimentally estimated molecular mass of the β-galactosidase subunit (Fantes and Roberts, 1973; Díaz et al., 1996). Using comparative genomics upon mining ortholog genes from the NCBI sequence databases, the gene models for the five GH2-GH35 loci in the annotation version 4 were manually corrected in order to correctly deduce the encoded proteins (see Supplementary Fig. S1). The conclusion remained that only the gene at locus AN3201 can code for a peptide of 120 kDal. This gene was assigned the abbreviation *bgaD*, for beta-galactosidase D, to differentiate it from the three *bga* loci previously described by Fantes and Roberts (1973).

Fig. 1 shows a phylogenetic study of the proteins deduced from the DNA sequences mined from the sequence databases using the BgaD protein as the TBLASTN bait. BgaD appears to be related to the structurally characterised β-galactosidase Lac4p from *Kluyveromyces lactis* (Poch et al., 1992) and more distally, to the two β-galactosidases from *E. coli* (Kalnins et al., 1983; Stokes et al., 1985). There are quite a few pezizomycotina that appear to harbour one or more genes for intracellular (pH neutral) β-galactosidase. The filamentous fungal enzymes cluster in two paralog clades while several species have presence in both clades, indicating that the paralogs have their origin in an ancient gene duplication. The BgaD-paralog *A. nidulans* protein is the product of the AN2463 locus, one of the GH2-coding genes on Chr. VII.

3.2. Identification of a potential lactose permease–β-galactosidase gene cluster

Screening the vicinity of locus AN3201, the first MFS sugar porter gene encountered was specified by locus AN3199, divergently transcribed from *bgaD* at 5.6 kb distance. To investigate the potential for a gene cluster, the MFS genes nearest to the genes of the eukaryotic β-galactosidases depicted in Fig. 1, were taken directly from the annotation of the corresponding genomes and the encoding proteins subjected to phylogenetic analysis (Fig. 2). All but four (among the latter, two duplicated genes and a pseudogene) filamentous fungal GH2 genes of which the products are clustered in the BgaD-ortholog clade in Fig. 1 have a MFS companion gene that appears to encode an ortholog of the *A. nidulans* sugar porter specified by locus AN3199. The eleven encoded proteins share at

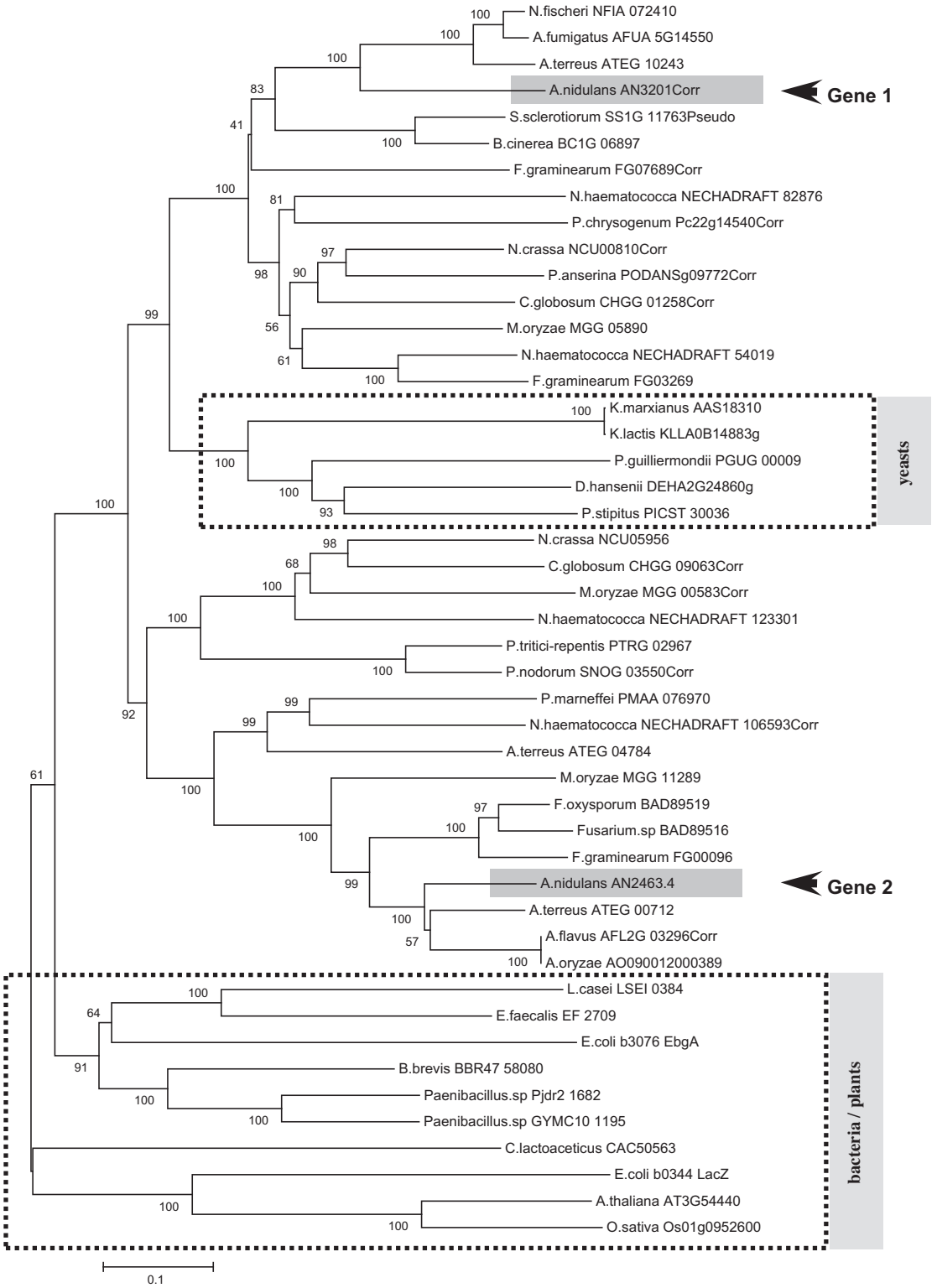

Fig. 1. Phylogenetic analysis of intracellular GH2 β-galactosidases from fungi. Proteins similar to the identified *A. nidulans* candidate β-galactosidase encoded at locus AN3201 were mined from the NCBI databases (as per February 2009) using TBLASTN. The tree was constructed using the neighbour-joining algorithm (as detailed in Section 2). Two clades of paralog fungal proteins occur, typified by the *A. nidulans* proteins specified by loci AN3201 (**Gene 1** = *bgaD*) and AN2463 (**Gene 2**), respectively. A subclade uniquely consistent of yeast proteins is boxed as is a selection of distally related bacterial and plant β-galactosidases. Numbers over nodes are bootstrap coefficients, determined from 1000 replica. Proteins are labelled by their annotated locus identifier. Miscalled gene models were manually corrected; the protein sequences thereof deduced are identified by their respective locus number tagged by the extension -Corr. For the reader's convenience, these manually corrected sequences are listed in Supplementary Document SDoc1. The corresponding nucleotide sequence data are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the accession numbers TPA: BK008491–BK008500.

least 61 % identity and all but one of the porter genes are transcribed divergently from the GH2 gene. Intergenic regions between the divergent partner genes range from 810 bp to 2034 bp, except in *A. nidulans*, where the genome annotation suggests the presence of another gene (locus AN3200) between *bgaD* and the sugar porter gene (see below). On the other hand, most genes of the 14 GH2 proteins constituting the BgaD-paralog clade in Fig. 1 (i.e., orthologs of the GH2 specified by locus AN2463) do not appear to have a conserved MFS-encoding companion, except in two species that do not feature representation in the BgaD-ortholog clade, *Pyrenophora tritici-repentis* and *Phaeophaeria nodorum*. The two dothideomycete MFS proteins are more similar to that specified by *A. nidulans* locus AN3199 than the three sugar porter companions identified in the genomes of the yeasts *Debaryomyces hansenii*, *Pichia stipitis* and *K. lactis*.

It would thus appear that a potential β-galactosidase/sugar porter gene cluster is conserved in *A. nidulans* and at least 15 other ascomycetes. We termed the MFS companion gene of *A. nidulans bgaD* at locus AN3199, *lacpA* (for lactose permease A).

3.3. Expression of the bgaD-lacpA gene cluster

To validate the *in silico* identified candidates for β-galactosidase and lactose permease, their expression profile was addressed at the transcript level. As shown in Fig. 3, *bgaD* is strongly induced in the presence of lactose or D-galactose, 6 h after a medium transfer of glycerol-grown mycelia while 26-h old lactose-grown mycelia yielded similar levels of induced transcription. A more modest response was visible for L-arabinose while no transcript could be seen upon transfer to D-glucose, glycerol, D-xylose or galactitol media. When either of the inducing sugars and glucose were simultaneously present at equimolar amounts, *bgaD* induction was completely repressed (results not shown). On the other hand, the *bgaD*-paralog gene on Chr. VII (locus AN2463) only responded to L-arabinose while for the two GH35 genes on Chr. VIII (loci AN0756 and AN0980), no responses could be observed (results not shown). *bgaD* (predicted molecular mass: 117.6 kDal, see below) is thus the only GH2–GH35 gene capable of producing a 120-kDal peptide, whose transcription profile is essentially identical to that of the ONP-Gal hydrolase described in the past (Paszewski et al., 1970; Fantes and Roberts, 1973; Fekete et al., 2002).

Next, the expression profiles of *bgaD* and *lacpA* were simultaneously addressed in wild-type and carbon catabolite-derepressed mutant (*creAΔ4*) backgrounds, using the same culture conditions as above. From Fig. 4, it appears that the two genes were coordinately expressed in both backgrounds, both with respect to induction as to repression of expression. This suggests that the two genes are functionally clustered.

Remarkably, carbon catabolite repression of the two genes appeared only partly relieved in the *creA* loss-of-function mutant. In the derepressed strain, a basal transcription level – almost as high as that in response to L-arabinose in the wild-type – occurred on those growth substrates that did not yield any visual expression in a wild-type background, including on the repressing sugar glucose. The wild-type response to L-arabinose appeared not additive to the basal level in *creAΔ4*. Moreover, any induced expression over the derepressed basal level in the presence of lactose/D-galactose and D-glucose could not be observed in the *creAΔ4* mutant suggesting the involvement of a CreA-independent mode of repression. Supplementary Fig. S2 shows that constitutive expression of *bgaD* in *creA* loss-of-function mutants concurred with constitutive β-galactosidase activity *in vivo*. The *creAΔ4* mutant and the wild-type reference were grown on minimal medium plates containing lactose, glucose or lactose/glucose as growth substrates, and X-Gal as chromogenic β-galactosidase substrate. While the wild-type strain only expressed hydrolase activity on lactose plates, the *creA* mutant colonies stained blue on all three media. Essentially identical results were obtained in a *creA*^{d}*30* mutant (results not shown).

Furthermore, the transcript study suggested that *bgaD* and *lacpA* could be expressed from a bidirectional promoter resident within the 5.6 kb separating the start codons. Unlike all other ortholog GH2-MFS gene clusters described above (cf. Fig. 2), the *A. nidulans* cluster is interrupted by an apparently complete gene (locus AN3200), putatively encoding another intracellular but much smaller GH2 glycosyl hydrolase (see Fig. 5 and Supplementary Fig. S1). Orthologs of this gene can found in a number of filamentous fungal genomes (see Supplementary Fig. S3). Addressing the expression of the intervening gene by means of Northern analysis, very weak signals could be detected indicating that it was constitutively expressed at low levels, i.e., not featuring any induction or repression responses under the conditions tested (results not shown). RT-PCR analysis of the same RNA samples (Fig. 5) confirmed that the gene at locus AN3200 is independently transcribed and sequence analysis of amplified cDNA (GenBank Accession JQ681216) confirmed the absence of two introns.

3.4. Structural analysis of the bgaD and lacpA genes

The complete genes for *bgaD* and *lacpA* were sequenced as well as cDNA generated from total RNA from lactose-induced biomass. Translation of the *bgaD* coding sequences yielded a 1043-amino acid long peptide with a deduced molecular mass of 117.1 kDal, in good correlation with the experimentally determined 120 kDal (Fantes and Roberts, 1973; Díaz et al., 1996). On the other hand, the 532-amino acid long peptide (59.1 kDal) encoded by *lacpA* appeared a typical MFS membrane protein with 12 transmembranal domains.

Unfortunately, it was not possible to analyse the *bgaD* gene in the lactose non-utilising β-galactosidase-negative (*lacG*) mutants selected by Gajewski et al. (1972). Only the β-galactosidase-defective mutation *bgaA0* (Fantes and Roberts, 1973) (see Supplementary Fig. S2) was available. The *bgaD* sequence in an outcrossed *bgaA0* mutant (GenBank GU129977) was identical to the corresponding *A. nidulans* genome sequences (GenBank AACD01000053) and that in strain R21, confirming that the *bgaA0* mutation is not located in the structural β-galactosidase gene. On the other hand, we sequenced the *lacpA* gene in one of the lactose mutants selected by Roberts (1963), carrying the uptake mutation *lacA1*. In contrast to what might be expected, the *lacpA* sequence in two independently obtained *lacA1* strains (including some 800-bp of promoter) turned out to be the same as the wild-type sequence (GenBank FJ647189). *lacA1* does not necessarily have to be localised within a sugar porter gene to result in an uptake phenotype. Still, the sequence data raised the question whether the *A. nidulans lacpA* gene encodes a physiologically relevant lactose permease.

3.5. Functional analysis of the lactose permease gene lacpA

The *lacpA* gene was deleted in a mutant lacking non-homologous end-joining activity (Δ*nkuA*) (see Section 2). All transformants tested were *lacpA* deleted (results not shown). However, when such gene-deleted mutants were tested for growth on minimal medium plates with lactose as the carbon source it was difficult to distinguish them from a number of wild-type strains (including the transformation host). Strains carrying the classical *lacA1* lactose uptake mutation consistently featured a phenotype, the residual growth of these mutants reflecting the ability of *A. nidulans* to use agar as an alternative, poor carbon source (Payton et al., 1976).

On the contrary, when growth on lactose was addressed in liquid minimal medium in shake flasks or fermenters, a clear phe-

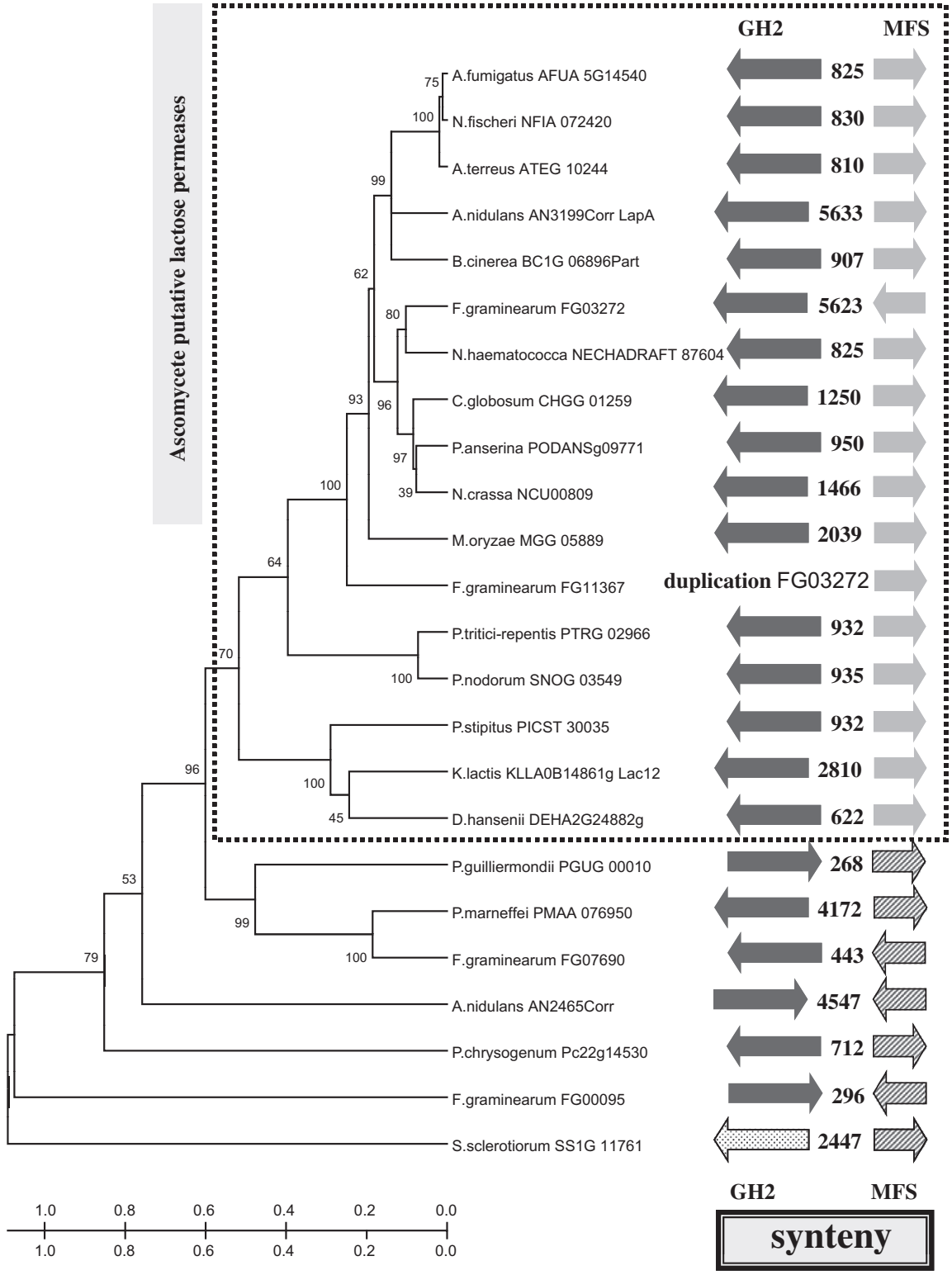

Fig. 2. Evolutionary relationships among the sugar porters, the encoding gene of which accompanies a eukaryotic GH2 β-galactosidase gene. Putative genes for MFS proteins nearest to the genes of the eukaryotic intracellar β-galactosidases in Fig. 1, were taken directly from the annotation of the corresponding genomes. For those β-galactosidase genes that do not appear to have a MFS-encoding companion within 10 kb distance (NB. All from the AN2463 clade), the products of the nearest MFS-encoding genes were discarded. The remaining 22 MFS proteins, all labelled with their respective annotated locus identifier, were subjected to phylogenetic analysis using the neighbour-joining algorithm with the divergence time set at an evolutionary rate of 1. Note that the *Botrytis cinerea* MFS gene at miscalled locus BC1G_06896 (tagged: -Part) is located at the end of a sequence contig and therefore, incomplete at its 3' end. At the right, the orientation of the transcription units of the neighbouring GH2 and MFS genes is depicted and the nucleotide distance separating the most proximal extremity of their respective coding regions (i.e. either the START- or the STOP codon) is given. The genes encoding those MFS proteins that cluster together with the lactose permease Lac12p from *K. lactis* (locus KLLA0B14861g) (Chang and Dickson, 1988) are depicted as light grey arrows: for these, divergent synteny with the neighbouring GH2 gene (dark grey arrows) is conserved except in *Fusarium graminearum*. Other MFS genes are represented by hatched arrows. Note that the GH2 gene in *Sclerotinia sclerotiorum* (dotted arrow) is a pseudo gene (at miscalled locus SS1G_11763); the MFS protein coded by the most proximal transporter gene (locus SS1G_11761) serves as a circumstantial outgroup for the phylogenetic tree.

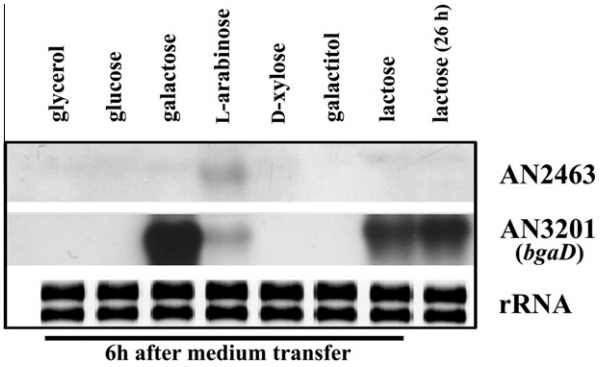

Fig. 3. Transcript analysis of the induction spectrum of two GH2 β-galactosidase paralog genes. Expression of the paralog genes specified by loci AN2463 (Chr. VII) and AN3201 (Chr. VI – *bgaD*) in response to simple sugars and related compounds was probed. Galactitol is the first intermediate of the alternative oxido-reductive pathway of D-galactose assimilation in *A. nidulans* (Fekete et al., 2004). In short, wild-type biomass (strain R21) was generated in shake flasks on glycerol as sole carbon source and then transferred for 6 h to fresh culture medium containing the various compounds tested or, alternatively, lactose-grown for 26 h. Total RNA was isolated from this biomass and subsequently denaturated, gel-separated and membrane-transferred as detailed in Section 2. The Northern blots were hybridised to gene-specific probes. As a control for the quality and quantity of the RNA, ribosomal RNA (28S and 18S) was visualised with ethidium bromide; the negative of the original image is shown at the bottom (rRNA).

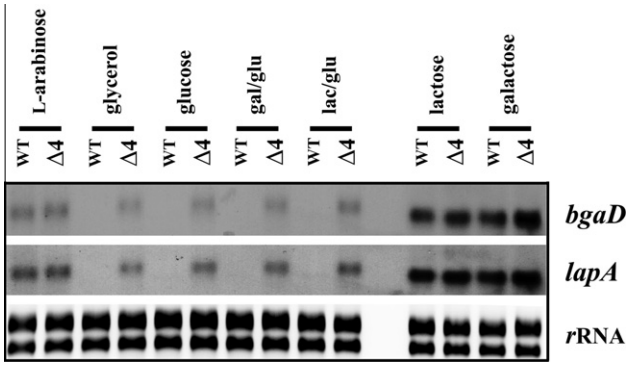

Fig. 4. Transcript analysis of the coordinated expression of *bgaD* (β-galactosidase) and *lacpA* (lactose permease). Co-expression of the *bgaD* (locus AN3201) and *lacpA* (locus AN3199) genes was addressed in a wild-type referent (R21) and the *creA* loss-of-function mutant *creAΔ*4 (V100). Total RNA and Northern blots were generated and hybridisations were carried out as described in the legend to Fig. 3.

notype was evident for *lacpA*-deleted strains (Fig. 6) Although capable of growing on lactose as the sole carbon source, they exhibited a considerably reduced growth rate (Fig. 6A), whereas a classical *lacA1* uptake mutant could not grow at all. All strains tested grew perfectly normal on D-galactose (Fig. 6B), D-glucose, L-arabinose, D-xylose, and glycerol as sole carbon sources in submerged culture (results not shown).

A. nidulans thus appears to express more than one lactose permease when growing on lactose, *lacpA* most likely encoding one of them. In *lacpA*-deleted strains from which the Δ*nkuA* deletion was crossed out (see Section 2), the discernible phenotype was maintained (results not shown). On the other hand, when a single copy of the *lacpA* gene was re-introduced in one of the *nkuA*-cured *lacpA*-deleted strains (see Section 2), the lactose growth characteristics were essentially identical to those of the wild-type R21. The *lacpA* re-introduction transformation also yielded transformants that had integrated more than one copy of *lacpA*. In lactose fermentation (Fig. 7), multicopy transformants grew considerably faster than single-copy strains and more biomass was formed by the time of carbon source exhaustion. Growth rate and biomass increased

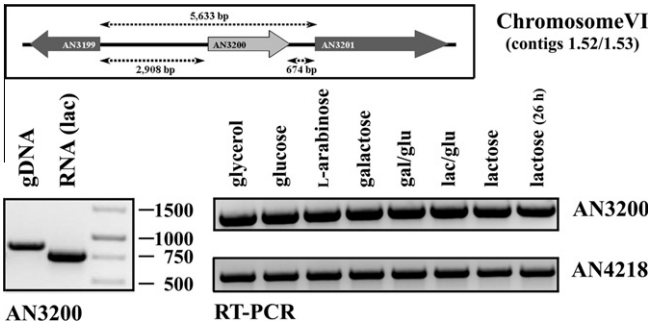

Fig. 5. Expression characteristics of the gene at locus AN3200, interrupting the continuity of the *bgaD*/*lacpA* gene cluster. The genetic context (loci AN3199 – *lacpA* and AN3201 – *bgaD*) is depicted above the semi-quantitative RT-PCR analysis of the wild-type expression of the GH2 gene residing on locus AN3200. Total RNA samples from strain R21 prepared for Northern analyses (cf. Fig. 3 and 4) were utilised as templates, as detailed in Section 2. Expression of the gene encoding the eukaryotic translation elongation factor 1-*alpha* component (i.e., the ortholog of the *Aspergillus oryzae tef1* gene (cf. Kitamoto et al., 1998), at miscalled locus AN4218.4) served as a control. As can be seen on the left, the amplification product from DNase-treated RNA was considerably smaller than that generated when genomic DNA was used to prime off AN3200-specific PCR, showing that this gene is transcribed under all conditions tested. cDNA sequences covering the complete coding region of the GH2 gene at locus AN3200 are available at GenBank under accession number JQ681216.

with the copy number, providing positive evidence that *lacpA* is physiologically relevant for growth on lactose and strongly suggesting that uptake is the rate-limiting step of lactose catabolism in *A. nidulans*.

To demonstrate that lactose uptake is affected in *lacpA*-deleted strains, mycelia of the wild-type referent and two gene-deleted strains were incubated with ^{14}C-radiolabelled lactose (Fig. 8). When the cultures were pregrown on lactose, all mycelia started to take up lactose immediately at a virtually constant rate, however, with the *lacpA*-deleted biomass the uptake rate was considerably lower. The uptake experiments confirmed that there is a second transport system in lactose-grown mycelia that would be responsible for about a third of the overall lactose uptake by the referent strain (when the initial substrate concentration was 100 μM). The reduced uptake coincided with the slower growth of Δ*lacpA* mutants on lactose in submerged cultures (see above), again suggesting that uptake is limiting. Concurrent with the absence of *lacpA* transcript on glycerol (see above), uptake of lactose by glycerol-grown wild-type mycelia only commenced after a lag period of about 1 h while glycerol-grown biomass of *lacpA*-deleted strains did not feature any lactose uptake of note during the time range of the uptake experiment (results not shown).

To see whether it would be possible to discern the uptake components from each other, lactose uptake was studied in the wild-type referent and *lacpA*-deleted strains at three different substrate concentrations (Table 1). The data show that the contribution of LacpA to the overall uptake of lactose increased with decreasing external substrate concentration, i.e., from less than 20% at 2 mM lactose to more than 65% at 100 μM. Such behaviour strongly suggests that *lacpA* codes for a lactose permease in *A. nidulans* and that the second uptake component expressed on lactose has a lower affinity for the disaccharide.

3.6. Formal confirmation that bgaD encodes β-galactosidase

The Results section gives a cronological account of our experimentation. Upon instigation of a reviewer, we deleted the *bgaD* gene (see Sections 2.5 and 2.6 for details). *bgaD* deletants and strains in which the functional gene was re-introduced in the gene-deleted background were tested on X-Gal plates. Supplemen-

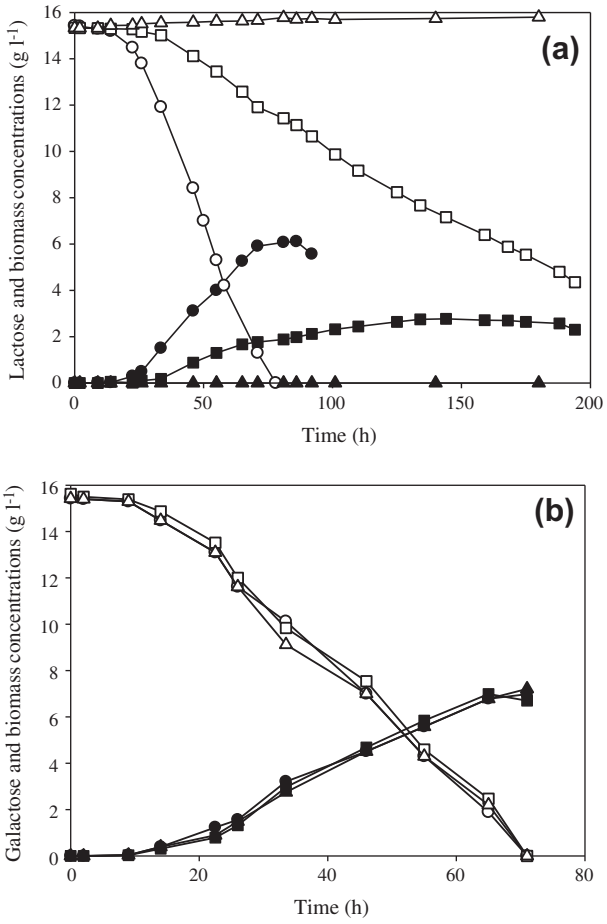

Fig. 6. Biomass formation and sugar consumption by *lacpA*-deleted strains during submerged growth on lactose or D-galactose. The growth and substrate consumption of two *lacpA*-deleted strains in lactose- (Panel A) and D-galactose (Panel B) fermentations were compared to those of the wild-type referent (R21) and Roberts' original *lacA1* lactose uptake mutant (G0103) (Roberts, 1963; Gajewski et al., 1972). See Section 2 for a detailed description. The biomass concentration (filled symbols) and the residual sugar concentration in the culture medium (open symbols) were plotted against time. The circles represent the data for the wild type, the squares those for one of the *lacpA*-deleted strains, and the triangles those for the *lacA1* mutant. The two panels give the averaged results for three independent fermentation experiments. In Panel A, the mean standard deviation for the lactose concentration was 5% and for the biomass concentration, 10%; the maximum deviations were 7% and 13%, respectively. In Panel B, the mean standard deviation for the galactose concentration was 5% and for the biomass concentration, 10%; the maximum deviations were 7% and 13%, respectively.

tary Fig. S4 (Panel A) shows that *bgaD* deletion led to a complete loss of the ability to hydrolyse the artificial substrate while β-galactosidase activity was re-gained upon *bgaD* re-introduction when lactose was the (inducing) carbon source. Blue staining could be detected earlier in colonies of *bgaD*-multicopy strains than in that of referent R21 and the stain was more intense in the former, strongly suggesting that β-galactosidase is overexpressed in strains carrying more than one *bgaD* copy. Essentially identical results were obtained on D-galactose (not shown). However, the lactose plates revealed a paradox as a growth phenotype was not apparent for *bgaD*-deleted strains. Growth experiments in liquid medium (Supplementary Fig. S4, Panel B) confirmed that a *bgaD*-deletant and two *bgaD*-multicopy transformants grew as fast on lactose as a wild-type. Thus although *bgaD* encodes the only enzyme acting on universally-employed chromogenic substrates generally regarded as β-galactosidase-specific, the gene is completely dispensable for utilisation of lactose in *A. nidulans*. Our observation validates the one from Fantes and Roberts (1973), albeit theirs was

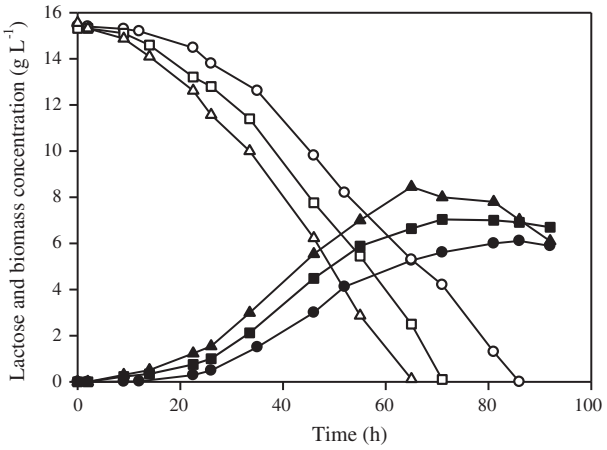

Fig. 7. Biomass formation and lactose consumption by transformant strains carrying more than one functional copy of the lactose permease (*lacpA*) gene. The growth and substrate consumption of transformant strains carrying multiple copies of the *lacpA* gene were compared with those of a transformant in which only one *lacpA* gene copy was re-introduced in a *lacpA* gene-deleted background. The experiments were performed and represented as described in the legend to Fig. 6. The circles signify the data for a strain in which one copy of *lacpA* was re-introduced, the squares those for a transformant carrying one extra copy of *lacpA*, and the triangles those for one carrying two extra copies. The plot gives the mean data of three independent fermentation experiments. The mean standard deviation for the lactose concentration was 5% and for the biomass concentration, 10%; the maximum deviations were 7% and 13%, respectively. Note that the behaviour of the one-copy transformant strain (circles) was essentially identical to that of the wild-type R21, for which the data are not shown.

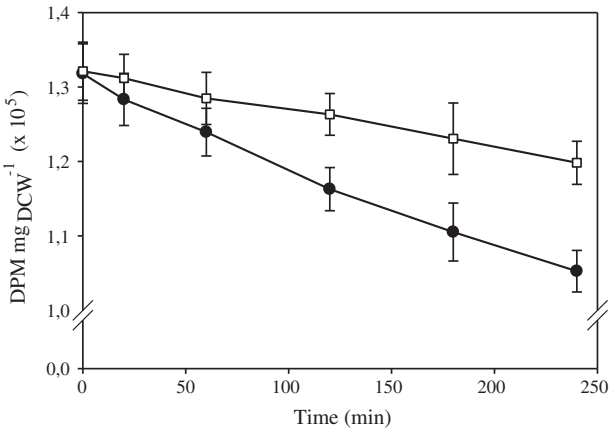

Fig. 8. Lactose uptake by wild-type and lactose permease (*lacpA*) gene-deleted strains. Lactose uptake was studied in lactose-grown mycelia from referent strain R21 and two *lacpA* gene-deleted strains as detailed in Section 2. The initial substrate concentration was 100 μM. The figure shows the averaged results of three independent repeats, with standard deviations given as error bars, and plots the residual label in the medium, given as d.p.m per mg DCW, versus the time elapsed since medium transfer. The open squares represent the data for one *lacpA*-deleted strain and the filled circles those for the wild-type referent.

Table 1
Lactose uptake in wild-type and *lacpA*-deleted *A. nidulans* strains at different substrate concentrations. The lactose uptake rate given is the mean of three independent uptake experiments; between brackets, standard deviation is expressed as a percentage of the average figure.

Strain	Lactose concentration (mM)	Specific uptake rate (μM min^{-1} $mg(DCW)^{-1}$)
R21 (wild type)	0.1	0.21 (9.5%)
	0.5	0.29 (10.3%)
	2	0.34 (7.3%)
EFLK_161 (Δ*lacpA*::Tr.*pyr4*)	0.1	0.07 (7.1%)
	0.5	0.20 (7.5%)
	2	0.28 (10.7%)

based on a mutation at a locus (*bgaA*; Chr. III) distinct from the structural β-galactosidase locus (*bgaD*; Chr. VI).

4. Discussion

4.1. Identification of a β-galactosidase/lactose permease gene cluster in A. nidulans

Lactose is a poor carbon source that is hydrolysed intracellularly in *A. nidulans*. In this communication, we identify a β-galactosidase/sugar porter gene cluster corresponding to annotated loci AN3201 and AN3199 on Chr. VI. We show that the *bgaD* and *lacpA* genes are coordinately regulated in response to inducing sugars as well as exhibiting a considerable constitutive expression level in *creA*-derepressed mutant backgrounds, despite the presence of an independently transcribed gene, putatively encoding another intracellular GH2, between them. The transcriptional behaviour of the intervening gene (locus AN3200) suggested that *bgaD* and *lacpA* have separate promoters rather than being controlled from a bidirectional promoter. Nevertheless, co-regulation of *bgaD* and *lacpA* at the transcript level is concurrent with the expression of glycosyl hydrolase activity against artificial β-galactosidase substrates.

Interestingly, the *bgaD* and *lacpA* genes appear to be subject to some form of CreA-independent carbon catabolite repression as lactose- or D-galactose induction over the constitutive basal transcript levels in *creA* mutants did not occur in the presence of equimolar amounts of D-glucose. This expression profile is distinct from the superinduction phenomenon that can be observed for induced expression of the alcohol dehydrogenase (*alcA*) and xylanase A (*xlnA*) genes, for which transcript levels are reached in *creA* loss-of-function mutants that are substantially higher than those that can be obtained in wild-type strains (Flipphi et al., 2003b). Superinduction is due to the lack of competition between the transcriptional activator (AlcR and XlnR, respectively) and the repressor CreA for promoter binding (Flipphi and Felenbok, 2004). It is therefore reasonable to presume that regulator competition – as a molecular mechanism for carbon catabolite repression – does not occur in the promoters of *bgaD* and *lacpA*. Instead, the role of CreA appears to be restricted to suppressing basal level transcription of both genes, allowing their non-induced expression upon carbon starvation.

4.2. Evolution of a β-galactosidase/lactose permease gene cluster in ascomycetes

The phylogenetic relations between ortholog GH2-MFS cluster genes (Fig. 1 and 2) suggest that this system has a long history in ascomycetes. The evolution of the cluster in pezizomycotina seems more complex than in the three yeasts. Not only the β-galactosidase gene appears to have duplicated in many species (cf. Fig. 1), the means to take up saccharide substrate(s) of such structurally related GH2 enzymes appear to have proliferated as well. In the *A. nidulans* genome, there are three MFS proteins specified – at loci AN1577, AN6831 and AN2814 – that are more similar to LacpA than any of these four *A. nidulans* proteins is to *K. lactis* Lac12p (Supplementary Fig. S5). Like has been observed for primary carbon catabolism (Flipphi et al., 2009), such duplication events are likely to contribute to the nutritional versatility of saprophytic and phytopathogenic filamentous fungi. Unlike is the case for, e.g., β-D-glucuronidase (Wenzl et al., 2005), there are no indications that ascomycete intracellular β-D-galactosidases have a recent bacterial origin. Instead, it is more likely that fungi that do not specify *bgaD* and *lacpA* ortholog genes in their genome, such as *A. niger* or *T. reesei*, have lost the cluster. In support of a thesis of duplication and adaptive loss of genes, all sequenced *Aspergillus* genomes specify an ortholog of AN1577 while, e.g., *A. aculeatus*, *A. carbonarius* and *A. niger* lack orthologs for LacpA, AN6831 and AN2814 (Supplementary Fig. S5). Gene loss can be seen at the present in *Sclerotinia sclerotiorum*, where the GH2 gene has regressed to a pseudogene.

4.3. LacpA is a physiologically relevant lactose transporter

We further characterised the product of the MFS-encoding cluster gene *lacpA* as a physiologically relevant lactose permease. *lacpA* deletion resulted in slower growth on lactose in submerged culture and in a reduced lactose uptake rate. *A. nidulans* features a multicomponent uptake system for the disaccharide lactose and LacpA is the transporter of higher affinity. On the other hand, LacpA is irrelevant for growth on D-galactose as sole carbon source, despite being induced by it. This contrasts with the situation in *K. lactis*, where the LacpA-ortholog Lac12p is the only lactose permease (Riley et al., 1987; Chang and Dickson, 1988; Lodi and Donnini, 2005) which was shown to contribute considerably to the uptake of the monosaccharide D-galactose (Riley et al., 1987; Boze et al., 1987).

The uptake experiments revealed that LacpA may have an important role in the onset of the utilisation of lactose in mycelia grown on other carbon sources. Wild-type mycelia grown on glycerol featured an adaptation period before they started to take up lactose. This is in line with the observation that *lacpA* was not expressed in the presence of glycerol while constitutively expressed in derepressed *creA*-mutant backgrounds that mimick carbon starvation (see Fig. 4). Apparently, basal *lacpA* expression is necessary for this adaptation as glycerol-grown biomass of *lacpA*-deletion strains did not take up lactose at all. However, *lacpA* is not necessary for germination and growth when lactose is the first growth substrate encountered. This might indicate that another lactose uptake component is expressed uniquely prior to or during spore germination, or that the system of lower affinity operative during growth on lactose (see Section 3) would be expressed transiently in germlings irrespective the presence or absence of the dissacharide. A precedent for the latter mode of expression has been described for the main permease of L-proline in *A. nidulans*, PrnB (Tazebay et al., 1997).

Increasing the gene dosis of *lacpA* – albeit encoding the permease of higher affinity – resulted in considerably higher biomass formation and a more rapid consumption when the initial lactose concentration was as high as 1.5 % (~38 mM) (see Fig. 7). An important conclusion of the current work with respect to industrial application and environmental issues related with the by-product lactose (e.g., whey bio-remediation or saccharification for biofuel production) is that uptake rather than hydrolysis is the rate-limiting step of lactose utilisation in the model fungus *A. nidulans*.

4.4. The possible nature of the lacA1 mutation

We identified the lactose/D-galactose-responsive *lacpA-bgaD* gene cluster on Chr. VI, characterised LacpA as as a physiological lactose permease and showed that BgaD is the *A. nidulans* β-galactosidase. However, we did not identify the gene that carries the lactose uptake mutation *lacA1* (Chr. VI; see Fig. 6) and, most likely, neither the gene harbouring the lost lactose non-utilising *lacG* mutations. *lacA1* may concern a regulatory mutation *in trans* affecting expression of all lactose uptake systems in *A. nidulans*, hence, the lactose-derived induction. *lacA1* does not appear to affect D-galactose-induced expression of β-D-galactosidase BgaD (Gajewski et al., 1972). One could speculate that *lacpA* may likewise be expressed in the *lacA1* background in response to D-galactose.

It is possible that the *bgaD*/*lacpA* cluster is subject to dual regulation, again, unlike the situation in the lactose-fermenting yeast *K.*

lactis, where lactose and D-galactose induction is mediated by one activator, the Galp4-ortholog Lac9p (Salmeron and Johnston, 1986; Wray et al., 1987). In *A. nidulans*, the lactose-derived induction could be mediated by a system-specific regulator (possible encoded by the gene carrying the *lacA1* mutation) while D-galactose-related regulation would be effected by a distinct activator protein, probably one that controls the downstream catabolism of the monosaccharide, GalR and/or GalX (Roberts, 1970; Christensen et al., 2011). Note that the zinc-cluster protein GalX bares substantial similarity to Gal4p/Lac9p. The regulatory mutation *galA1* affecting expression of enzymes involved in D-galactose assimilation is allelic to *galX* (locus AN10543). The *A. nidulans* genome specifies two *galX* paralogs, at loci AN8111 (Chr. II) and AN4785 (Chr. III; not linked to *galX*). However, the lactose non-utilising mutation *lacA1*, which affects lactose uptake and may be involved in the lactose-derived induction of *bgaD*/*lacpA* expression, maps to Chr. VI.

4.5. Lactose utilisation in β-galactosidase-defective mutants of A. nidulans

In *Kluyveromyces lactis*, the clustered structural genes *LAC4* and *LAC12* – the yeast's orthologs of *bgaD* and *lacpA*, respectively – are both necessary and sufficient for lactose utilisation (Sheetz and Dickson, 1981; Riley et al., 1987; Lodi and Donnini, 2005). It may seem confusing that BgaD is dispensable in *A. nidulans*, while LacpA is a physiological lactose transporter. However, the phylogenetic studies of both proteins strongly suggested that the GH2/MFS cluster is ancestral to the ascomycete lineage and evolved (see above) long before the emergence of mammals, the principal sources of milk sugar. The function of the intracellular β-galactosidase in saprophytic and phytopathogenic filamentous fungi – and in extension, a more ancestral cluster function – is likely related with the release of β-galactoside and/or α-L-arabinopyranoside units from polymers of vegetal/fungal origin, as reflected by, e.g., the constitutive expression of *bgaD*/*lacpA* in carbon-derepressed backgrounds and the L-arabinose response of *bgaD* and its paralog at locus AN2463. It is more than reasonable to presume that lactose is a (poor) growth substrate for *A. nidulans* – a soil-borne saprophyte – and other filamentous fungi, by coincidence rather than by design.

To grow on lactose, *A. nidulans bgaD*-deletants must express a more aglycon-specific galactosyl hydrolase (i.e., inert towards X-Gal and ONP-Gal). The related species *A. niger* has lost the *bgaD*/*lacpA* cluster (see above) and its growth on lactose most likely depends on an extracellular GH35 β-galactosidase encoded by the *lacA* gene (Kumar et al., 1992). Expression of *A. niger lacA* in *Saccharomyces cerevisiae* is sufficient to allow the yeast to grow on lactose or whey permeate. However, the mere existence of the lactose uptake mutation *lacA1* suggests that the alternative lactose hydrolase in *A. nidulans* is an intracellular enzyme. If a secreted hydrolase would be involved, the fungus would not need to take any lactose from the medium. On the contrary, the *lacA1* mutant did not grow at all on lactose in liquid cultures while it grew perfectly normal on D-galactose or D-glucose. In concordance, we found that the GH35 genes specified by loci AN0756 and AN0980 – the ortholog of *A. niger lacA* and its sole paralog in *A. nidulans*, respectively – were not expressed on lactose or D-galactose (or under any other of the tested conditions; see Section 3) in the wild-type.

We want to emphasise that it has not been shown that β-galactosidase BgaD would be irrelevant for lactose assimilation. Purified BgaD does hydrolyse lactose (Díaz et al., 1996). Fantes and Roberts (1973) demonstrated that lactose hydrolysis was considerably reduced in D-galactose-induced *bgaA0* biomass where ONP-Gal hydrolase activity was virtually absent. The residual activity they measured with lactose as the substrate may be taken as an indication for the existence of an alternative hydrolase. Note that this enzyme may be poorly expressed on D-galactose.

We have shown that lactose uptake rather than hydrolysis is rate-limiting and overexpression of BgaD from multiple *bgaD* copies did not lead to faster growth. It is therefore possible that the extant *in vivo* activity of the alternative hydrolase is sufficient to maintain a wild-type growth rate on lactose in β-galactosidase-defective strains.

Note added in proof

In this study, manually corrected amino acid sequences have been used (listed in Supplementary document SDoc1). For the β-galactosidases, the corresponding nucleotide sequence data are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the accession numbers TPA: BK008491–BK008500.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.03.001.

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