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Characterization of a second physiologically relevant lactose permease gene (lacpB) in Aspergillus nidulans

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Characterization of a second physiologically relevant lactose permease gene (*lacpB*) in *Aspergillus nidulans*

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Abstract

In *Aspergillus nidulans*, uptake rather than hydrolysis is the rate-limiting step of lactose catabolism. Deletion of the lactose permease A (*lacpA*) gene reduces the growth rate on lactose while its overexpression enables faster growth than wild type strains are capable of. We have identified a second physiologically relevant lactose transporter, LacpB. Glycerol-grown mycelia from mutants deleted for *lacpB* appear to take up only minute amounts of lactose during the first 60 hours after a medium transfer, while mycelia of double *lacpA*/*lacpB* deletant strains are unable to produce new biomass from lactose. Although transcription of both *lacp* genes was strongly induced by lactose, their inducer profiles differ markedly. *lacpB* responded also strongly to beta-linked glucopyranose dimers cellobiose and sophorose, while these inducers of the cellulolytic system did not provoke any *lacpA* response. On the other hand, *lacpA* but not *lacpB* expression was high in D-galactose cultures. In a *lacpA*-negative background, *lacpB* was overinduced by cellobiose in comparison to wild type; consequently, cellobiose uptake was faster and biomass formation accelerated in *lacpA* deletants. In contrast, in *lacpB* knockout strains, growth rate and cellobiose uptake were considerably reduced relative to wild type, indicating that the cellulose- and lactose catabolic systems employ common elements. Nevertheless, our permease mutants still grew on cellobiose which suggests that its uptake in *A. nidulans* prominently involves hitherto unknown transport systems.

Introduction

Dairy industry generates prodigious amounts of liquid waste (Gänzle *et al.*, 2008). Some 85 % of the components of the milk destined for cheese manufacture are eventually discarded as a watery lactose-rich by-product called whey (Marwaha & Kennedy, 1988). Generated at over 1.5 million tons per year worldwide (Roelfsema *et al.*, 2010), raw, untreated whey poses huge environmental challenges, as current and past wastewater treatment technologies are expensive. An alternative to disposal is utilizing the whey residue in downstream (industrial) processes by which value-added products are or can be manufactured (Panesar & Kennedy, 2012). A prime example is industrial-scale fermentation biotechnology for which whey is traditionally considered a cheap and abundant growth substrate and nitrogen source for micro-organisms, fungi in particular (Coghill & Moyer, 1947; Silva *et al.*, 2009; Panesar *et al.*, 2006; Kumari *et al.*, 2011; Aghcheh & Kubicek, 2015).

Lactose (1,4-O-β-D-galactopyranosyl-D-glucose; milk sugar) is the main carbohydrate in whey. Mammalian milk is the only source of lactose in nature. For most micro-organisms that can hydrolyze it into D-glucose and D-galactose, lactose is a slowly assimilated, gratuitous carbon source not encountered in their natural habitats. This characteristic considerably facilitates the (over)production of secondary metabolites and hydrolytic enzymes by saprophytic- and plant-pathogenic fungi that apparently grow under laboratory conditions on lactose by chance rather than by design. Operation of the *LAC* regulon of the lactose-fermenting yeast *Kluyveromyces lactis* is a paradigm for transcriptional control in lower eukaryotes (Cardinali *et al.*, 1997; Baruffini *et al.*, 2006; Rigamonte *et al.*, 2011). Nevertheless, several key aspects of fungal lactose metabolism in less substrate-adapted ascomycete filamentous fungi (including potent cell factories) are poorly understood (see e.g., Seiboth *et al.*, 2007; Karaffa *et al.*, 2013). To optimize fermentation processes that use whey

residue, and to further its use in second-generation biofuel generation and its removal from contaminated soil and water (bioremediation), we study lactose catabolism in several filamentous fungi including the genetic model *Aspergillus nidulans*, a soil-borne saprophyte.

Two strategies have been described for the catabolism of lactose in fungi: extracellular hydrolysis and subsequent uptake of the resulting monomers, i.e., D-glucose and D-galactose, and uptake of the disaccharide followed by intracellular hydrolysis (reviewed by, *e.g.*, Seiboth *et al.*, 2007). In analogy with the *K. lactis* lactose assimilation system (*e.g.*, Gödecke *et al.*, 1991; Diniz *et al.*, 2012), we identified and characterized two clustered, divergently transcribed genes in *A. nidulans*, encoding an intracellular β-galactosidase of the Glycoside Hydrolase family 2 (*bgaD*) and a lactose permease belonging to the Major Facilitator Superfamily of transmembrane proteins (*lacpA*), respectively (Fekete *et al*., 2012). These two genes were expressed to basal levels in carbon-derepressed (*creA*^{d}) mutant backgrounds, even when the strongly repressing sugar D-glucose was the only growth substrate present. By creating deletion mutants, we provided evidence that *bgaD* is the only hydrolase in *A. nidulans* that acts on the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) – generally regarded as a typical albeit artificial β-galactosidase substrate. Moreover, we demonstated that LacpA – the first physiologically relevant, fungal lactose permease described outside the *Saccharomycetales* – mediates high affinity uptake of lactose. Such a β-galactosidase/lactose permease gene cluster was found conserved in at least 15 other filamentous ascomycetes (*Pezizomycotina*) (Fekete *et al*., 2012).

Furthermore, evidence was provided that transport rather than hydrolysis is the limiting step of lactose catabolism in *A. nidulans*, as overexpression of *lacpA* allows multiple copy transformants to grow considerably faster on the disaccharide than wild-type strains can. Although LacpA is responsible for a considerable part of the lactose uptake in this fungus, *lacpA* knock-out strains still grow on it (Fekete *et al*., 2012) implying that at least one

additional uptake system must be operative. In this report, we identify and functionally analyze a second physiologically relevant lactose permease gene in *A. nidulans* that we have named *lacpB.*

Materials and methods

A. nidulans strains, media and culture conditions

A. nidulans strains and transformants used in this study are listed in **Table S1**. Minimal media (AMM2) for shake flask and bioreactor cultivations (the latter henceforth referred to as 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 1.5 % (w/v) initial concentration unless stated differently. Cultures were inoculated with 10^6 *A. nidulans* conidia per ml of medium. Shake-flask cultures were incubated at 37 ºC in 500 mL Erlenmeyer flasks containing 100 mL of culture medium in a rotary shaker at 200 rpm.

Fermentations were carried out in a 2.5 L glass vessel (Sartorius, Göttingen, Germany) with a culture volume of 2 L, and equipped with one six-blade Rushton disc turbine impeller. Operating conditions were pH 6.5, 37 ºC, and 0.5 vvm (volumes of air per volume of liquid per minute). The dissolved oxygen level was maintained at 20 % saturation and was controlled by means of the agitation rate. To minimize medium loss, the waste gas was cooled in a reflux condenser connected to an external cooling bath (4 ºC) before exiting the system.

For induction experiments (also referred to as expression- or transcript analysis), replacement cultures were used for which mycelia were pregrown for 24 h in AMM2 medium containing glycerol as the carbon source, and harvested by filtration over a sintered glass funnel. After thoroughly washing the biomass with cold sterile water, mycelia were transferred to flasks with carbon-free, fresh AMM2 and were pre-incubated for 1 h in a rotary shaker at 200 rpm, after which the carbon sources to be tested were added to the cultures in

final concentrations up to 25 mM. Samples were taken after 3, 6 and 12 h of further incubation to assess inductory ability. Preliminary trails had established that 3 h of contact is the time lapse in which maximal induced transcript levels were achieved, with a minimal variation in the biomass concentration.

Lactose uptake experiments

Conidiospores were inoculated overnight on 15 g/L glycerol as the carbon source. Grown mycelia were transferred first to AMM2 medium containing lactose (15 g/L) as a sole carbon source for 24 h. Mycelia were subsequently harvested by gentle filtration over sterile cheese cloth, thoroughly washed with carbon-free AMM2 and resuspended in 500 mL Erlenmeyer-flasks containing AMM2 to yield a final biomass concentration of 1 g/L. Lactose was administered to final concentrations of 0.2 mM, 0.5 mM and 2 mM, and the cultures were incubated for further 6 h in a rotary shaker (37 ºC, 200 rpm) to monitor sugar consumption. Samples were withdrawn at regular intervals, cellular debris was spun down in an Eppendorf centrifuge (10.000 g, 5 min), and residual lactose in the supernatant was determined by High Performance Liquid Chromatography (HPLC; see below). D-Glucose in a final concentration of 2 mM was used for the control cultures. Biomass-corrected uptake was expressed in μmoles per gram of dry cellular weight (DCW). Specific uptakes rates were calculated from the specific uptake plotted against time, and were expressed in μmoles of lactose per minute and gram of DCW.

Classical genetic techniques and transformation

Conventional genetic techniques were employed to exchange markers by meiotic recombination (Clutterbuck, 1974). Progeny of sexual crosses was tested for known auxothrophies using standard techniques.

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

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 NucleoSpin Plant II, whereas total RNA was isolated with NucleoSpin RNA Plant (both kits from Macherey-Nagel, Düren, Germany).

Northern and Southern blot analysis

Standard procedures (Sambrook & Russell, 2001) were used for the quantification, denaturation, gel separation and nylon blotting of nucleic acids, and the hybridization 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 (**Table S2**) off R21 genomic DNA. Hybridization was visualized with Lumi-Film Chemiluminescent Detection film (Roche Applied Science). cDNA was synthesized from 1 µg of DNase I-treated total RNA using Oligo(dT) as a primer and Moloney murine leukemia virus reverse transcriptase.

Generation of knock-out mutants strains

A gene deletion cassette was constructed *in vitro* according to the double-joint PCR method (Yu *et al.*, 2004). The cassette consisted of 840 bp of the terminal, noncoding regions of *A.*

nidulans lacpB flanking the functional *A. fumigatus pyroA* gene involved in pyridoxine biosynthesis (Nayak *et al.*, 2006). Oligonucleotide primers used are listed in **Table S2**. Protoplasts of *A. nidulans* pyridoxine-auxotroph strain TN02A3 were transformed with 10 μg of the linear deletion cassette. This transformation host greatly facilitates the obtention of gene knockouts due to the absence of a functional Nonhomologous End-Joining machinery. Pyridoxine-prototroph transformants were probed for the absence of *lacpB* coding sequences by PCR, primed off genomic DNA using gene-specific primers.

For the creation of the double transporter mutant (*lacpA*/*lacpB*), the same *in vitro lacpB* replacement construct as described above was introduced into one of the pyridoxine-auxotroph *ΔlacpA*/Δ*nkuA* strains. Selected pyridoxine-prototroph, double deletant strains were verified by Southern blot analysis and then crossed out to rid the *nkuA* deletion. Throughout this work two independent double transporter mutants were tested.

Re-introduction of lacpB into gene-deleted backgrounds

A characterised first generation deletant of *lacpB* was crossed with strain RJMP155.55. Pyridoxine-prototroph and riboflavin-auxotroph offspring was verified by PCR for the presence of the *nkuA* gene. A functional *lacpB* gene was amplified off *A. nidulans* R21 genomic DNA using specific primers (**Table S2**). 10 μg of the amplification product was co-transformed with 1 μg of pTN2 (carrying the *A. fumigatus riboB* gene encoding a protein involved in riboflavin biosynthesis; Nayak *et al.*, 2006) into one of the Δ*nkuA*-cured, second generation gene-deleted strains. Among the riboflavin-prototroph transformants, the presence of the re-introduced gene was probed by PCR. The *lacpB* copy number was subsequently estimated by Southern blot analysis, and selected strains that had re-acquired functional *lacpB* in one or more copies, were phenotypically characterized.

Analytical methods

DCW was determined from 10 ml culture aliquots. The biomass was harvested and washed on a preweighted glass wool filter by suction filtration, washed with cold tap water 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 more than 14 %. D-Glucose, D-galactose and lactose were determined by HPLC with refractive index detection as described by Fekete *et al*. (2002).

Reproducibility

All the analytical and biochemical data presented are the means of three to five independent experiments (biological replicates). Data were analyzed and visualized with SigmaPlot (Jandel Scientific), and for each procedure, standard deviations (SDs) were determined. The significance of changes in biomass and in residual lactose concentration in the growth medium of mutant- or complemented deletant strains relative to the control cultures, was assessed using Student's t-test with probability (*p*) values given in the Results section.

Chemicals

Except where specified, chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich Kft. (Budapest, Hungary).

Bio-informatics methods (cf. Fig. S1)

A. nidulans lactose permease A (LacpA) (Fekete *et al.*, 2012) was used as the query in TBLASTN mining of genes encoding structurally related major facilitator superfamily transmembrane proteins in some three dozen species of the genus *Aspergillus*, from nucleotide databases available at the National Center for Biotechnology Information (NCBI)

and the US Dept. of Energy Joint Genome Institute (JGI). Gene models and products were deduced manually. The 92 peptidic sequences most similar to *A. nidulans* LacpA were aligned with MAFFT version 7 (Katoh & Toh, 2010) using the G-INS-i algorithm (trained on global homology) and a BLOSUM 45 similarity matrix. Protein input is available from the corresponding author upon request. The alignment was curated with Block Mapping and Gathering using Entropy (BMGE version 1.12; Criscuolo & Gribaldo, 2010) employing a BLOSUM 35 similarity matrix and a block size of 3, yielding 477 informative residues per protein. A maximum likelihood tree was then calculated with PhyML version 3.0 applying the WAG substitution model (Guindon et al., 2010) and drawn with FigTree (available at http://tree.bio.ed.ac.uk/software/figtree). Approximate likelihood ratio tests (Anisimova & Gascuel, 2006) were calculated integrally by PhyML using Chi2-based parametrics; aLRT values (0–1) are given at the connecting nodes in the tree.

Results

In silico identification and expression verification of putative lactose permease genes

The genome of *A. nidulans* harbours over 100 predicted genes that encode transmembrane proteins belonging to the Sugar Porter Family of the Major Facilitator Superfamily (MFS) (Wortman *et al.*, 2009). To track down structurally related permeases, we examined the phylogenetic relations amongst a selection of *Aspergillus* MFS proteins (**Fig. S1**). The genes for the LacpA orthologs and for the seven structural paralog proteins most similar to *A. nidulans* LacpA were mined from publicly accessible nucleotide databases upon a TBLASTN search (Altschul *et al.*, 1997) and the respective gene models and protein products were subsequently deduced. The resulting 92 proteins were used as input to build a Maximum Likelihood tree. Lactose permease LacpA (protein_1 in **Fig. S1**) is specified in only 14 genomes, while a closely related protein (protein_2) is encoded in all but three of the 31 genomes. Nevertheless, no species were found to harbor all selected MFS genes: *A. ustus* and *A. versicolor* carry six of the paralog genes. On the other hand, *A. glaucus* and *A. rubrum* (section Aspergillus) lacked all eight (not shown). The three structurally related *A. nidulans* MFS proteins mined among the seven most similar *Aspergillus* paralogs of LacpA, specified by genes at the annotated loci AN1577, AN6831 and AN2814, were selected as candidate lactose permeases to subject to a biological validation.

The expression of the three candidate genes in response to the presence of lactose and – as a negative control of expression – D-glucose was addressed by Reverse Transcriptase PCR. As shown in **Fig. 1**, the gene specified at locus AN2814 was well expressed in the presence of lactose 6 h after a medium transfer of glycerol-grown mycelia, compared to glucose. On the other hand, the gene at locus AN1577 gave a much weaker signal on lactose, while the third paralog gene (locus AN6831) appears to be modestly transcribed on glucose

rather than specifically on lactose. Moreover, the related fungus *Penicillium chrysogenum* – *Pencillium* is the sister genus of *Aspergillus* in the family of the *Aspergillaceae* – reportedly has the intracellular GH2 β-galactosidase ortholog of *A. nidulans* BgaD but its genome does not specify transporters ortholog to LacpA and that encoded at *A. nidulans* locus AN1577 (Jónás *et al.*, 2014). In conclusion, *in silico* analysis of the *A. nidulans* genome sequences followed by a restricted test of expression of three selected candidates suggested that the gene at locus AN2814 encodes a LacpA-paralog MFS protein that may contribute to uptake of lactose by *A. nidulans*. We named this putative second lactose permease gene, *lacpB*.

Confirmation that lacpB encodes a lactose permease, and functional analysis of the gene

To verify that *lacpB* indeed encodes a functional lactose permease, the gene was knocked out. *LacpB* deletants and strains in which the functional gene was re-introduced in the gene-deleted background carrying one or more (i.e., 2 and 5) copies at ectopic loci were subjected to phenotypic analysis. As **Fig. 2** shows, *lacpB* deletion led to a sharp decrease in the amounts of lactose taken up when compared to the wild-type control strain, and no (new) biomass was formed before the third day of fermentations with lactose as the sole carbon source. On the other hand, no growth phenotype was visible on D-glucose and D-galactose (data not shown). Somewhat unexpectedly, lactose uptake as well as fungal biomass formation set off approximately three days after inoculation at a rate comparable to that of the wild-type strain at the rapid growth stage of the cultivation, and continued until the available lactose was fully consumed (**Fig. 2**). Nevertheless, final maximal biomass concentrations remained significantly (p<0.1%) lower than in the reference culture. This pattern of delayed carbon uptake and growth during lactose fermentation of the *lacpB*-deletant strains appeared to occur regardless of the initial substrate concentration in the range of 1 to 20 g/L (~ 3 – 58 mM; data not shown). At 50 and 100 g/L (~ 0.15 M and 0.3 M) of initial lactose concentrations – which

is the usual range in mammalian milk – even the wild-type strain appeared growth inhibited, a phenomenon that may indicate that *A. nidulans* has little tolerance against high concentrations of lactose at the stage of germination (**Fig. S2**).

Uptake is rate-limiting for wild-type lactose assimilation in *A. nidulans* (Fekete *et al.*, 2012). Investigation of the complemented *lacpB* knock-out strains revealed that the copy number of *lacpB* was proportional to the efficiency of lactose uptake and subsequent biomass formation (**Fig. 3**). Since single-copy retransformants behaved essentially identical to the wild-type strain, the results in strains with multiple copies of *lacpB* implied that LacpB is physiologically relevant. Multi-copy strains displayed a significantly (p<1%) enhanced lactose uptake rate, as evident from the accelerated disappearance of lactose from the growth medium. Concomitantly, biomass formation was also significantly (p<1%) faster, the growth rate increasing with the increasing number of *lacpB* copies. As a consequence, final biomass concentrations achieved by the multi-copy strains were significantly (p<1% for the 2-copy, p<0.1 % for the 5-copy) higher than those of the wild-type or the (mono-copy) retransformant strains.

To demonstrate that lactose uptake is affected in *lacpB*-deleted strains, pre-grown mycelia of the reference- as well as the mutant strains were incubated with low concentrations (0.2 mM, 0.5 mM, 2 mM) of lactose (**Fig. 4**). At each concentration, specific lactose uptake of the wild-type strain was higher than that of deletant, but lower compared to the multi-copy mutants (**Fig. 4**). Moreover, lactose uptake was significantly (p<1%) faster in the strain that carries five copies of the *lacpB* gene compared to the one harboring two copies.

To appreciate whether the contribution of LacpB to the overall uptake of lactose varied with the external substrate concentration, lactose uptake was studied in the wild-type referent, a *lacpB*-deletant as well as in the two-copy and five-copy *lacpB*-retransformant strains (**Table 1**). The data show that while lactose uptake of the *lacpB*-deletant strain at 0.2 and 0.5 mM

(initial) external lactose concentrations was approximately 14% and 11% of that of the wild-type strain at those concentrations, respectively, it was about 22% at 2 mM. All these observations are direct evidence that *lacpB* encodes a physiologically relevant lactose permease in *A. nidulans*. Moreover, LacpB appears crucial to initiate and sustain uninhibited growth on lactose at concentrations up to 20 g/L without a long lag phase.

Lactose uptake is mediated by two permeases in Aspergillus nidulans

In a previous publication we demonstrated that the *A. nidulans lacpA* gene encodes a lactose permease that is physiologically relevant albeit only responsible for part of the total uptake (Fekete *et al.*, 2012). The new data on the function of the paralog permease LacpB described above prompted us to investigate whether it may account for the remaining uptake potential in *lacpA* deletant strains. To this end, *lacpB* was deleted in a *lacpA*-negative background, giving rise to *lacpA/B* double lactose permease mutants. Phenotypic analysis of the strains revealed that conidial germination was completely inhibited in liquid media with lactose as the sole carbon source at concentrations that allow growth of single *lacpB* deletants (results not shown). Moreover, glycerol-grown double mutant mycelia transferred to lactose-AMM2 were unable to form new biomass even after a prolonged fermentation time and unsurprizingly, lactose consumption could not be detected (**Fig. 5**). The specific lactose uptake rates – assessed with the same means as in the single deletion and retransformant mutants – did not reflect any statistically relevant uptake by the double *lacp* permease mutant (**Table 1**).

The expression profile of lacpB is different from that of lacpA

To investigate the regulation of *lacpB,* its expression profile was addressed at the transcript level. As shown in **Fig. 6(a)**, *lacpB* in the wild-type strain was strongly induced in the presence of lactose as early as 3 h after medium transfer of glycerol-grown mycelia. In the

presence of D-galactose, a modest response was evident at 3 h, and no response could be observed at the later time points (6 and 12 h). In contrast, *lacpA* responded to the monosaccharide D-galactose as least as strongly as to lactose at 3 h although later on, transcript levels on D-galactose faded (**Fig. 6b**). Another difference between the expression of the two *lacp* genes was observed in the presence of L-arabinose: as reported previously, L-arabinose provokes induction of *lacpA* (Fekete *et al.*, 2012) but *lacpB* expression could not be observed. As could be expected, the strongly repressing monosaccharide D-glucose did not provoke transcript accumulation from either *lacp* genes at any of the three time points, and this was also the case for the pentose D-xylose and the D-glucose C-2 epimer D-mannose (**Fig. 6a, b**).

In the *Aspergillus* genome database (AspGD; http://www.aspergillusgenome.org), *lacpB* (AN02814) was auto-annotated as a putative cellobiose (4-O-β-D-glucopyranosyl-D-glucose) transporter gene (Cerqueira et al., 2013), as it encodes the structural ortholog of the *Neurospora crassa* CDT-1 transporter (locus NCU00801), a permease that was shown to transport cellobiose when expressed in yeast (Galazka *et al.*, 2010). We tested the physiological response of both *A. nidulans lacp* genes to the presence of cellobiose in the growth medium. As **Fig. 6(a, b)** shows, *lacpB* was strongly induced in the wild-type strain particularly at 3 and 6 h, while no induction could be observed for *lacpA* using Northern blot transcript analysis.

Cellobiose is a low molecular mass inducer of fungal cellulolytic systems (for a review, see Seiboth *et al.*, 2007). To verify whether its inducing effect on *lacpB* transcription is related to lignocellulose breakdown, we monitored the responses of the two *lacp* genes in the presence of another beta-linked glucopyranose dimer, sophorose (2-O-β-D-glucopyranosyl-D-glucose), a potent cellulolytic inducer in *T. reesei* but not in *A. niger* (Sternberg & Mandels, 1980; Gielkens *et al.*, 1999). As shown in **Fig. 6(c)**, the induction

response to sophorose was very similar to that of cellobiose, i.e. no transcript could be detected for *lacpA*, while *lacpB* was strongly induced, particularly at 3 h and 6 h after medium shift.

The two differentially expressed lactose permeases appear to be jointly responsible for lactose uptake in *A. nidulans* under physiological conditions (see above). To probe whether their expression is coordinated, we assessed how one lactose permease gene reacts to the loss of the other in single permease deleted backgrounds (i.e., either Δ*lacpA* or Δ*lacpB*) (**Fig. 6a, b).** *lacpB* appeared better induced on D-galactose while its response seemed prolongated on lactose and, interestingly, on cellobiose in the *lacpA* deletion strain. Similarly, the *lacpA* response to D-galactose was seemingly extended in the absence of *lacpB* although its expression in the presence of lactose appeared to be delayed. *lacpA* remained irresponsive to cellobiose in the *lacpB* deletant. These observations point towards possible interplay between the lactose permeases and/or their regulatory circuits.

LacpB may be a common component of lactose- and cellulose catabolism in A. nidulans

The finding that *lacpB* is strongly induced by cellobiose led us to investigate whether the respective knock-out strains exhibit a growth phenotype on this disaccharide, when serving as the sole carbon source. Controlled fermentations were performed to obtain kinetic profiles for biomass formation and cellobiose consumption. As shown in **Fig. 7**, the *lacpA*- and *lacpB* single- as well as the double *lacpA/lacpB* mutant displayed a clear cellobiose phenotype relative to the wild-type reference and the monocopy re-transformant strain (NB. These latter two strains again behaved essentially the same). In a *lacpA*-negative background, cellobiose was consumed more rapidly (p<0.1%) (**Fig. 7a**), and consequently, fungal biomass formation was significantly (p<1%) faster in comparison to the control (**Fig. 7b**). This correlates with the *lacpB* expression profile observed in this mutant (see above). On the contrary, the growth

rate and cellobiose consumption of the *lacpB*-deletant and the double permease mutant strains were significantly (p<1%) lower. These results collaborate with our transcript analyses and demonstrate that the product of *lacpB* is involved in the transport of cellobiose in *A. nidulans*; LacpB should therefore be considered an important component of the cellulolytic system of this fungus. It must be noted, however, that growth of the *lacpB* deletion mutants on cellobiose was reduced, but not eliminated: each of our permease mutants germinated, grew and sporulated on this disaccharide. These results strongly suggested that cellobiose uptake in *A. nidulans* prominently involves additional, hitherto unknown transport systems.

Discussion

As an abundant by-product of cheese manufacture, lactose has long been used as a growth substrate for the production of fungal metabolites such as penicillin and hydrolytic enzymes (Roelfsema *et al.*, 2010). However, from several perspectives, it is an unusual disaccharide. It exclusively occurs in mammalian milk where it makes up 2–8 % of the dry weight. Saprophytic fungi and fungal plant, entomo- and mycopathogens do not encounter lactose in their natural habitats and thus – in the absence of tailor-made enzymes and transporters – they are either unable to utilize it or do so at a slow rate. That said, the sugar transport potential of the soil-borne saprophyte *A. nidulans*, whose genome is predicted to harbour over 400 genes encoding putative MFS 12-transmembrane domain proteins (Wortman *et al.*, 2009) appears diverse enough for some uptake of lactose, enabling its subsequent intracellular hydrolysis into D-glucose and D-galactose, which are both natural carbon sources for most fungi. Indeed, *Aspergillus* versatility is handsomely illustrated by the variation of occurance of LacpA and the seven MFS proteins structurally most similar to it (documented in **Fig. S1**).

In this work, we demonstrated that *A. nidulans* has two permeases, LacpA and LacpB, that transport the gratuitous carbon source lactose under standard laboratory conditions (i.e., concentrations up to 2 % w/v). We cannot exclude the existence of other permeases that have the biochemical capability to transport lactose over the plasma membrane. However, the expression of such transporter(s) is apparently not appropriate to initiate germination and/or enable sustained growth on lactose in the absence of both LacpA and LacpB.

In general, conidiospore germination efficiency decreases with increasing osmolality of the medium (for a quantitative analysis in *A. niger*, see Wucherpfennig *et al.*, 2011). Lactose is amongst the least osmotically active sugars (Mustapha *et al.*, 1997). Despite of this fact, we found that wild-type *A. nidulans* could not germinate at or over 5% (w/v) lactose. This is remarkable since other Aspergilli of industrial interest are routinely grown on substrates containing well over 10 % of carbohydrate (e.g., Karaffa *et al.*, 2015).

Based on the residual lactose uptake of the respective single permease deletants, LacpB may have a senior function during germination and early growth on lactose, while LacpA appears accessory. However, only deletion of both *lacp* genes resulted in a complete cessation of growth on lactose. This is in contrast to the situation in the lactose fermenting yeast *K. lactis* which expresses only one physiological lactose transporter, Lac12 (Riley *et al.*, 1987; Lodi & Donnini, 2005).

The difference in the response to cellobiose as well as to its positional isomer sophorose was absolute as we found that *lacpB* transcripts were produced abundantly upon induction by these two beta-linked glucopyranose dimers, while we could not observe any expression of *lacpA*. Cellobiose and lactose are structurally similar epimers only differing at the C4 position of the nonreducing monosaccharide unit, and our results suggest that "gratuitous" lactose catabolism is intimately connected to the fungal cellulolytic system. This functional link has previously been described for *T. reesei*, for which lactose is the only

soluble carbon source that induces cellulase- and hemicellulase enzymes in economically viable industrial growth conditions (Bischof *et al.*, 2013). The lactose transporter Crt1 (annotated protein identifier Trire2:3405) appears to be essential for growth on lactose and its deletion prevents lactose-induced cellulase gene expression (Ivanova *et al.*, 2013; Zhang *et al*., 2013; Cai *et al.*, 2015). The structural LacpB ortholog from *N. crassa* (locus NCU00801) has been described as a potent cellobiose transporter (Galazka *et al.*, 2010). In contrast, the *A. niger* genome does not specify orthologs for *lacpB* and *lacpA*, and interestingly, sophorose does not induce cellulolytic genes in this species (Gielkens *et al.*, 1999). Furthermore, this *Aspergillus* species is known to hydrolyze lactose exclusively extracellular with GH35 glycoside hydrolases (see e.g., Nevalainen, 1981; O´Connell & Walsh, 2010).

Our current results suggest that LacpB appears to be integral to cellulose catabolism. Meanwhile, LacpA may primarily be involved in the degradation of type I arabinogalactan, a pectic plant cell wall polymer. *A. nidulans* produces arabinogalactan endo-β-1,4-galactanase (EC 3.2.1.89; locus identifier AN5727), an extracellular GH53 that ultimately yields D-galactose and β-1,4-galactobiose (Michalak *et al.*, 2012; Otten *et al.*, 2013). We hypothesize that LacpA is a permease for β-1,4-galactobiose that facilitates its intracellular hydrolysis by the GH2 β-1,4-galactosidase BgaD and may transport lactose as a side activity, as these two disaccharides differ at the C4 of the reducing monosaccharide unit only. In support of this thesis, we had previously shown that D-galactose – the monomeric end product of extracellular endo-β-1,4-galactanase activity – is a potent inducer of the clustered *lacpA* and *bgaD* genes in *A. nidulans*, and that two putative extracellular GH35 β-1,4-galactosidase genes are not expressed in this fungus under the tested conditions (Fekete *et al.*, 2012). In conclusion, the two-pronged uptake system of lactose in the soil-borne saprophyte *A. nidulans* may represent an unexpected interface of two important hydrolytic systems necessary to degrade plant cell walls and to use them as growth substrate.

Acknowledgements

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Legends to the Figures:

Figure 1. Expression of three putative lactose permease genes in response to lactose visualized by semi-quantitative RT-PCR. For experimental details, see Material and Methods section. The strongly repressing carbon source D-glucose serves as a control condition: *lacpA* and *bgaD* expression are repressed by glucose even when lactose is also present in the growth medium (Fekete *et al.*, 2012). By contrast, the gene at locus AN3200 – located between *lacpA* and *bgaD* – was previously shown to be constitutively expressed to low levels under a number of conditions, including on D-glucose (Fekete *et al.*, 2012).

Figure 2. Time-profile of growth (circles) as well as residual lactose concentrations (squares) in a batch fermentation of the *A. nidulans* wild-type strain (filled symbols) and a *lacpB* deletion mutant (open symbols) in media initially containing 15 g/L lactose. Conidiospores were used as inoculum. Lactose was the sole carbon source.

Figure 3. Kinetics of lactose uptake (filled symbols; **panel A**) and biomass formation (open symbols; **panel B**) of the monocopy retransformant strain "AOEF011.1" (squares) compared to the 2-copy (circles) and 5-copy (triangles) *lacpB* strains "AOEF011.9" and "AOEF011.7", respectively. Note that the monocopy retransformant essentially behaves like wild type control strain R21.

Figure 4. Specific lactose uptake in the wild-type (circles), a *lacpB* deletant (squares) as well as the 2-copy (triangles) and 5-copy (diamonds) *lacpB* multicopy strains in media initially containing 0.2 mM (**panel A**), 0.5 mM (**panel B**) or 2 mM (**panel C**) lactose. **Panel D**: specific D-glucose uptake in the same strains in a medium initially containing 2 mM D-

glucose. Note that the Y-axis are differently scaled in Panel A, Panel B and Panel C-D due to the different initial substrate concentration in each experiment. As a consequence, one cannot visually compare the apparent uptake rates between different panels.

Figure 5. Time-profile of growth (circles) as well as residual lactose concentration (squares) in a batch fermentation of an *A. nidulans lacpA*/*lacpB* double deletion mutant in media initially containing 15 g/L lactose. Glycerol-germinated pregrown mycelia were used as inoculum. Identical results were obtained for other independently obtained double lactose permease mutants.

Figure 6. Transcript analysis of the *A. nidulans lacpB* (**Panel a**) and *lacpA* (**Panel b**) gene in wild-type- and in *lacpA*-negative backgrounds. Ribosomal RNA (28 S and 18 S) was visualized with ethidium bromide and is shown in the top- and the bottom panels as a control for RNA sample quality and quantity. **Panel c**: expression of the *A. nidulans lacpB* (**left**) and *lacpA* (**right**) genes in response to sophorose.

Figure 7. Time-profiles of residual cellobiose concentration (filled symbols; **Panel a**) and growth (open symbols; **Panel b**) in batch fermentations of the monocopy *lacpB* retransformant (circles), the *lacpA* deletant (squares), the *lacpB* deletant (triangles) and the *lacpA*/*lacpB* double permease deleted (diamonds) *A. nidulans* strains. The monocopy retransformant essentially behaves like wild-type control strain R21. Media were inoculated with conidiospores.

Table 1. Lactose uptake in wild-type *A. nidulans* and in *lacpB* deletant-, *lacpB* multicopy- and *lacpA*/*lacpB* double mutant strains at different substrate concentrations. Differences between data from the monocopy retransformant and the wild-type control R21 were statistically irrelevant (p <0.1%) (not shown).

***A. nidulans* strain**	**Lactose concentration (mM)**	**Specific uptake rate (μM lactose/min/g_{DCW})**
Wild-type (R21)	0.2	0.523 ± 0.49
	0.5	0.702 ± 0.68
	2.0	0.893 ± 0.84
Δ*lacpB*	0.2	0.075 ± 0.01
	0.5	0.077 ± 0.01
	2.0	0.196 ± 0.03
AOEF011.9 (2 *lacpB* copies)	0.2	0.510 ± 0.06
	0.5	0.834 ± 0.07
	2.0	1.411 ± 0.12
AOEF011.7 (5 *lacpB* copies)	0.2	0.651 ± 0.05
	0.5	1.067 ± 0.12
	2.0	1.468 ± 0.14
Δ*lacpA*/Δ*lacpB*	0.2	< 0.05
	0.5	< 0.05
	2.0	< 0.05

Figure 1.

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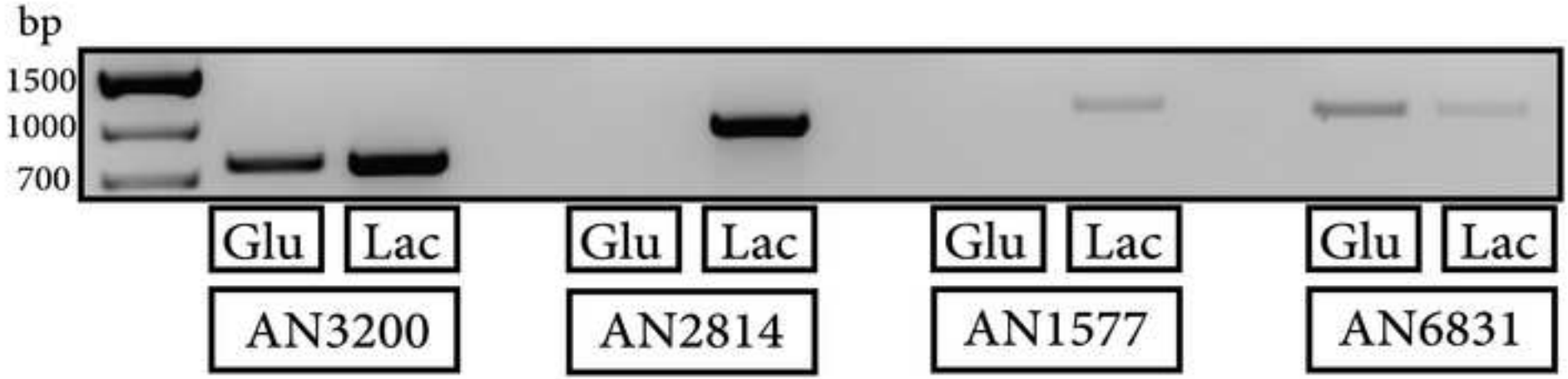

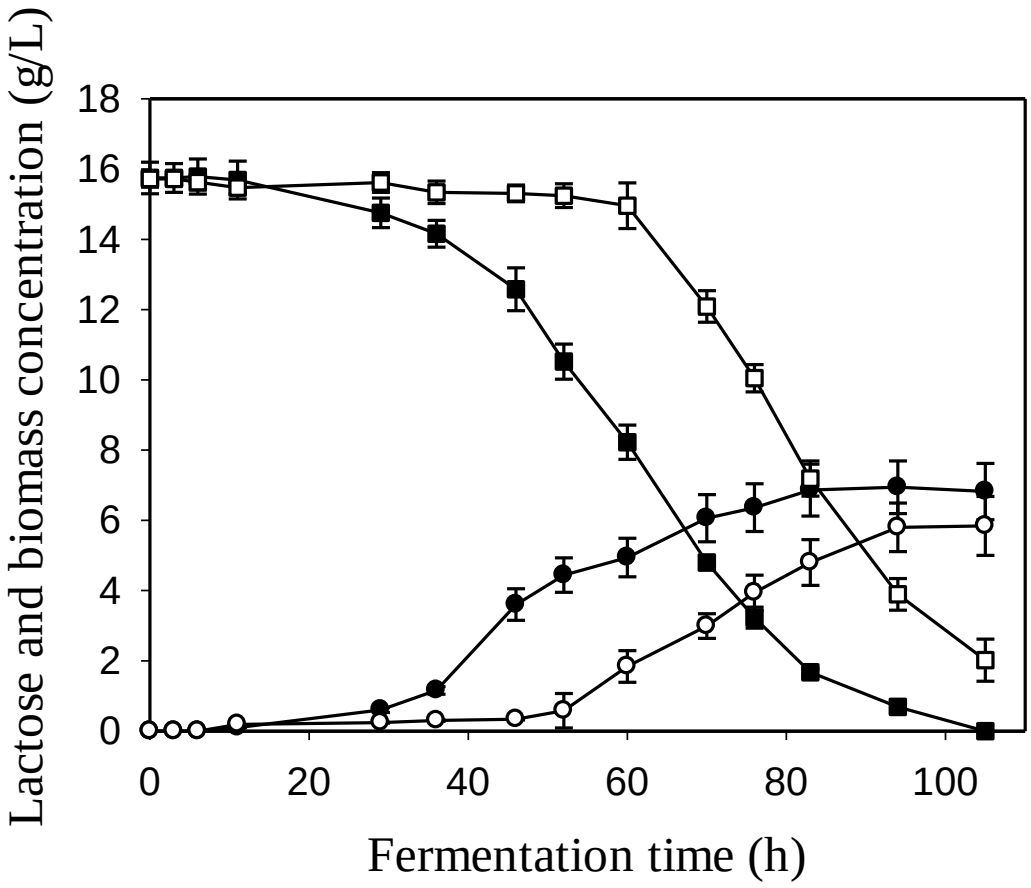
Lactose and biomass concentration (g/L)
18
16
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10
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4
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0
20
40
60
80
100
Fermentation time (h)

Figure 2.

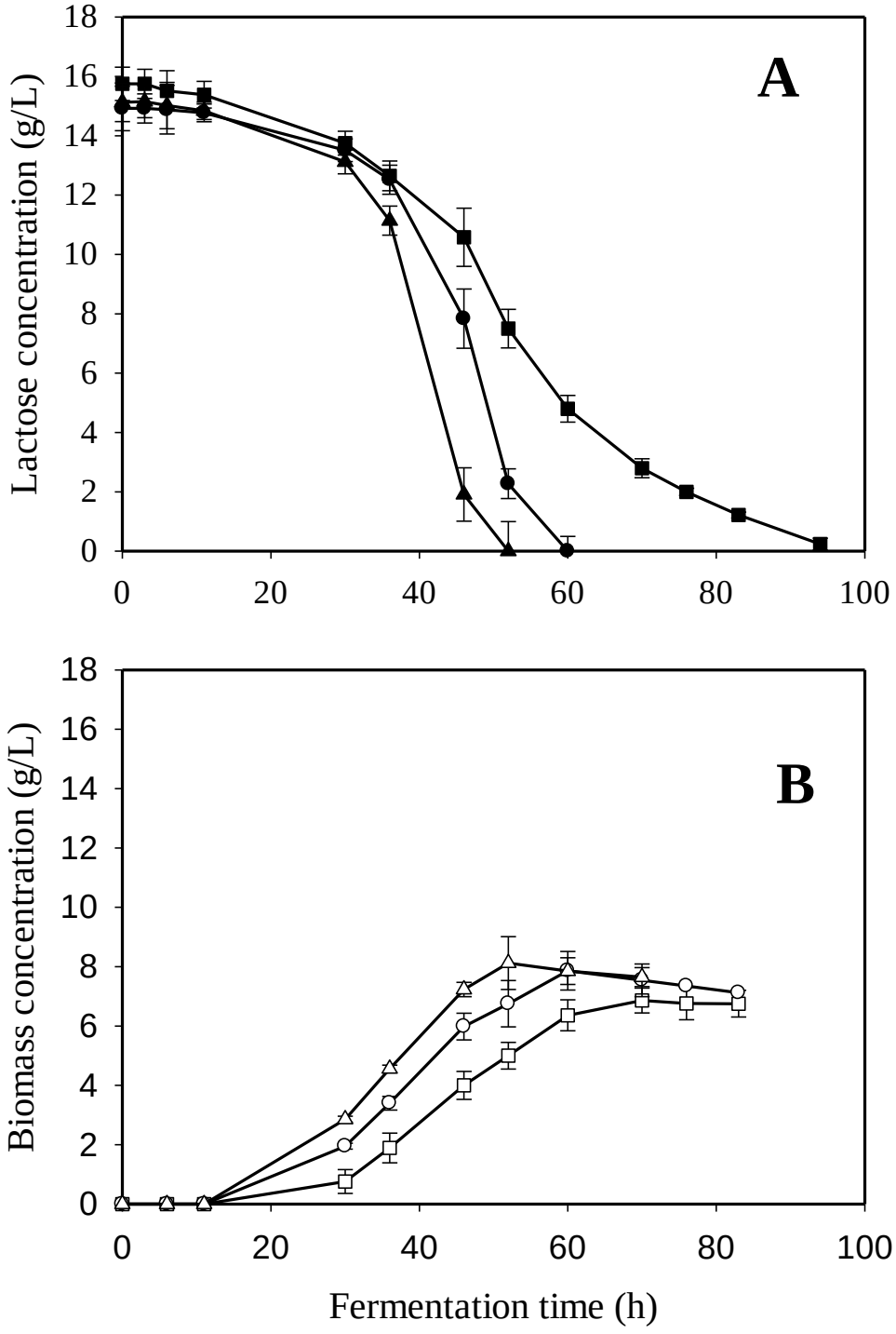

A
Lactose concentration (g/L)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
B
Biomass concentration (g/L)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
Fermentation time (h)

Figure 3.

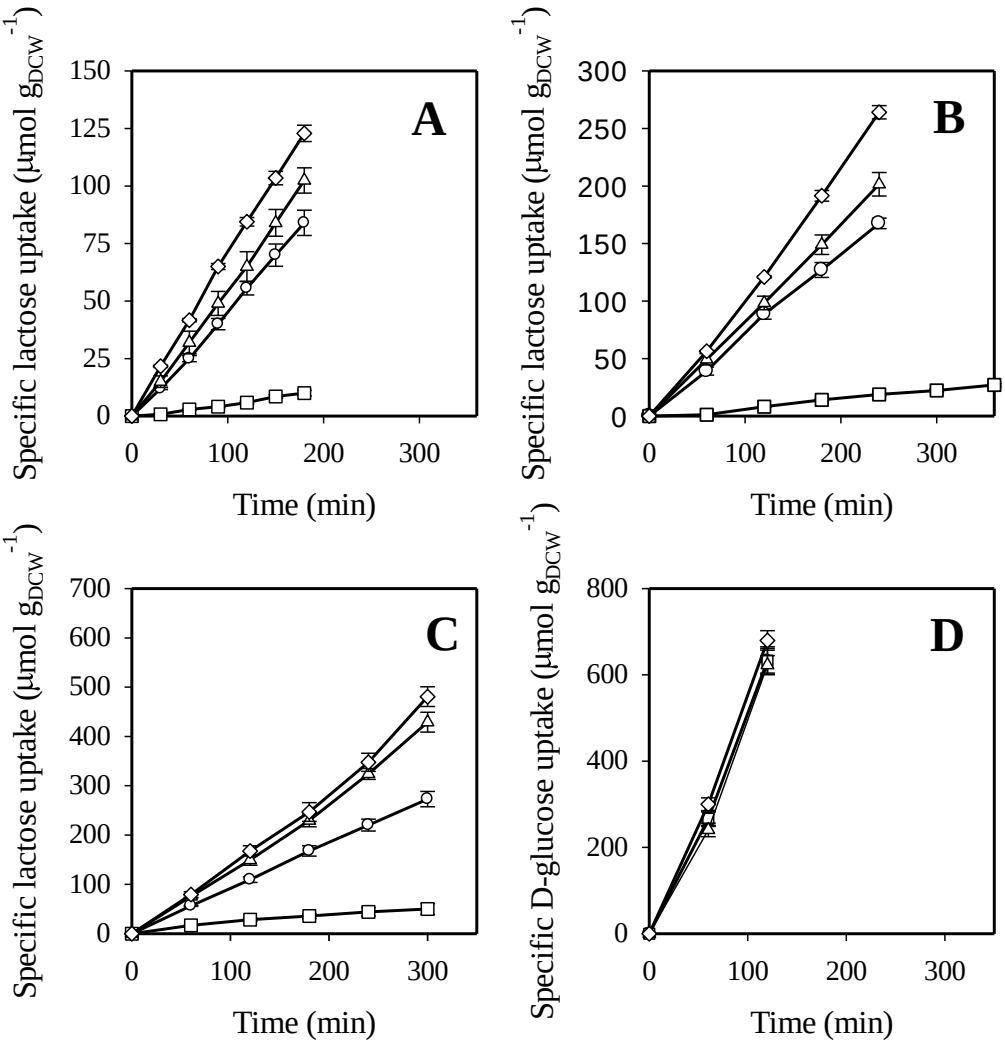
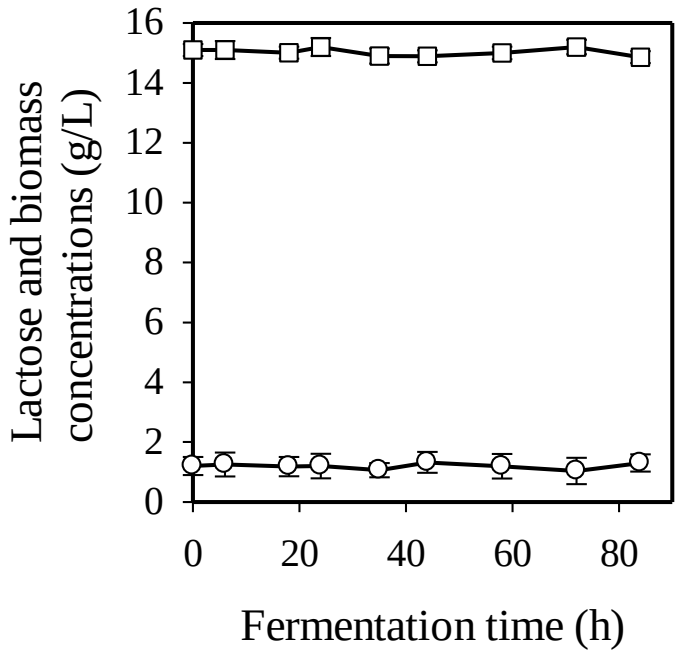


Figure 4.


Lactose and biomass concentrations (g/L)
16
14
12
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2
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40
60
80
Fermentation time (h)

Figure 5.

Figure 6.

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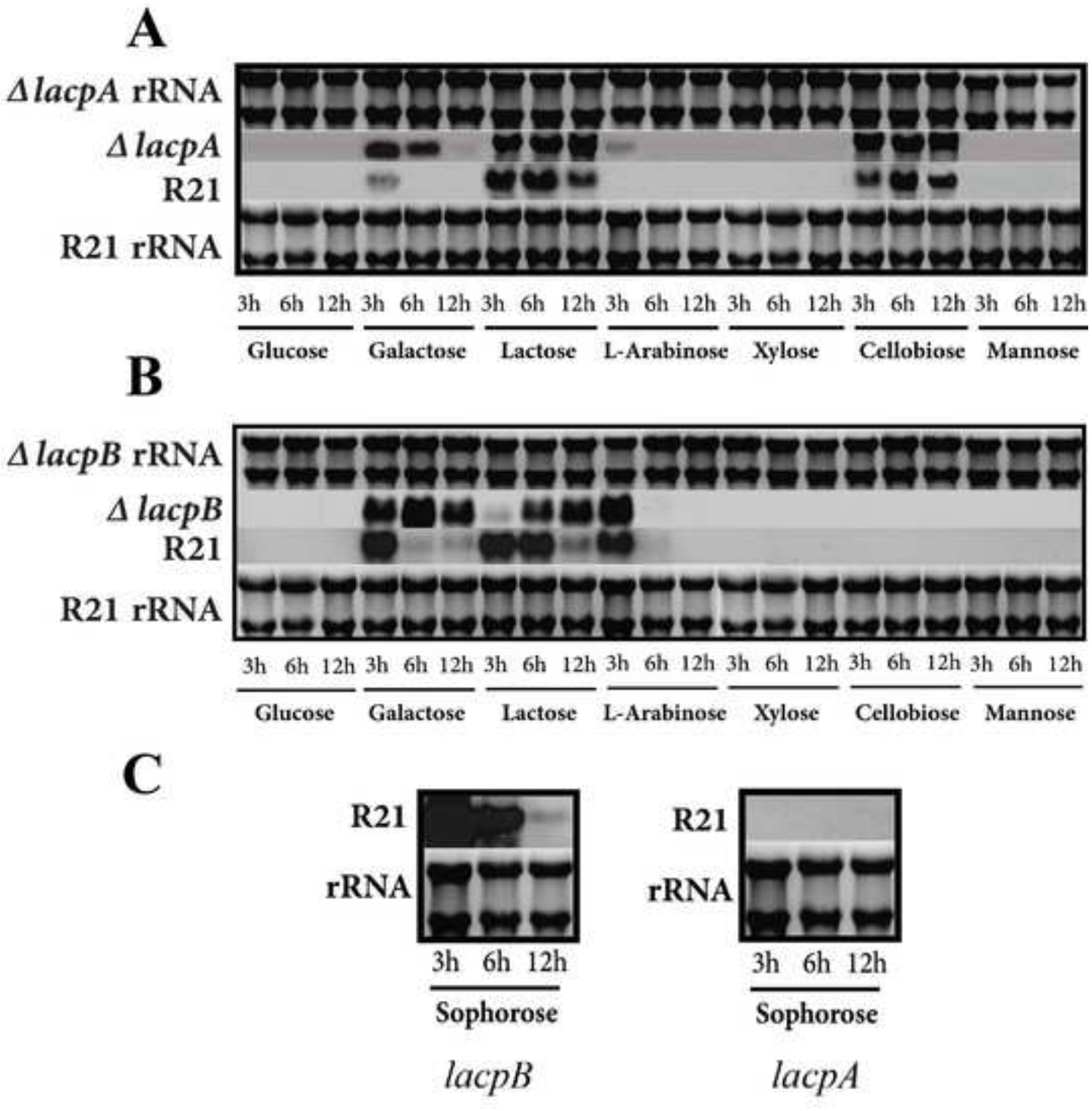

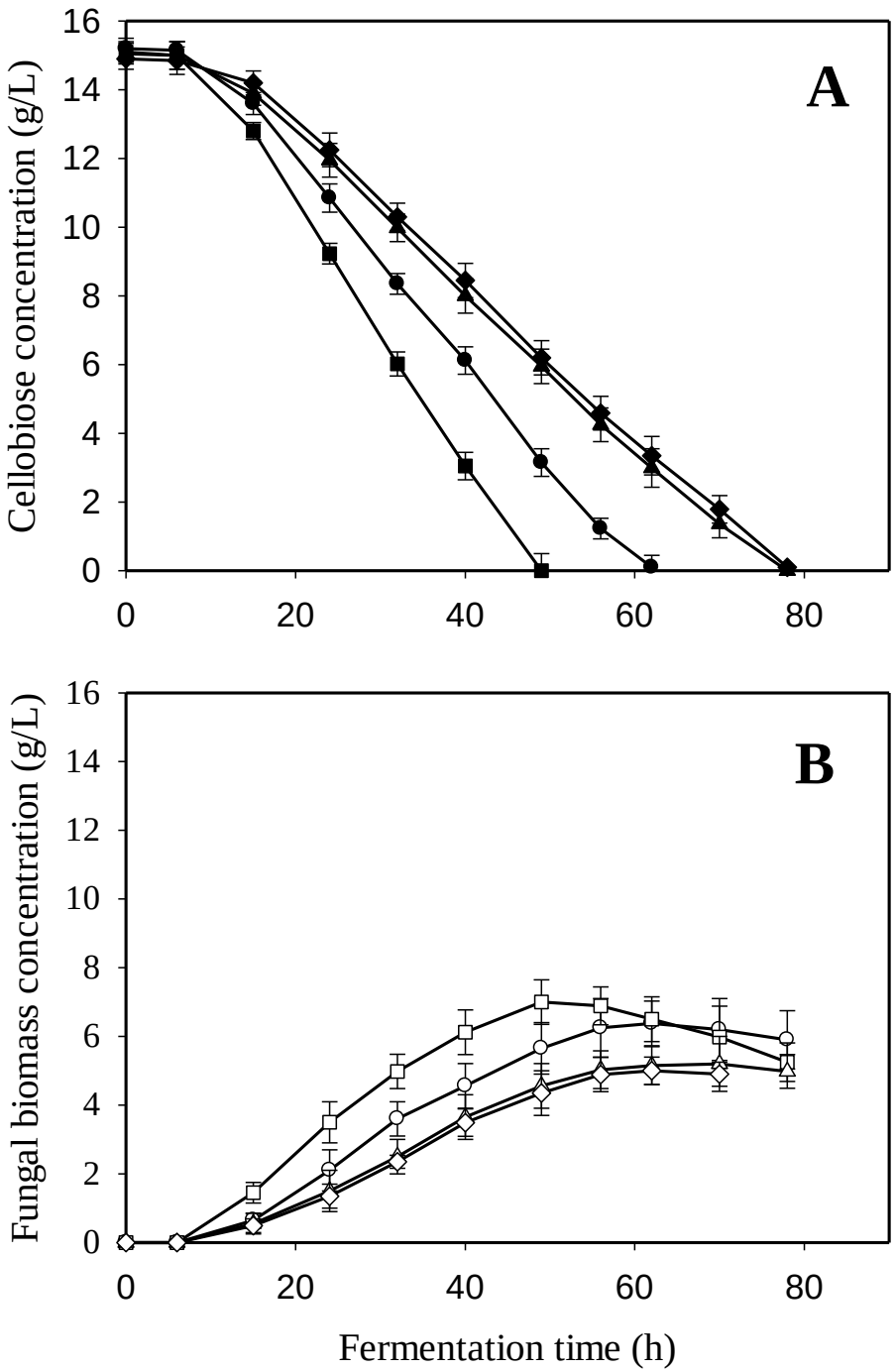
A
B
Cellobiose concentration (g/L)
Fungal biomass concentration (g/L)
Fermentation time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80

Figure 7.

Supplementary Material

Supplementary Table S1: *Aspergillus nidulans* strains used in this study

Strain	Genotype	Reference
R21 [a]	*pabaA1; yA2; veA1*	Fantes and Roberts (1973)
TN02A3 (FGSC A1149)	*pyrG89*; *pyroA4;* Δ***nkuA****::argB; veA1*	Nayak *et al.* (2006)
EFLK_161/9	(*pyrG89*); *pyroA4;* **Δ*nkuA*** *::argB*; *veA1*; **Δ*lacpA*** *::*Tr.*pyr4* [b] (uridine prototroph)	Fekete *et al.* (2012)
RJMP155.55	*riboB2; wA3; veA*$^{+}$	Gift from Prof. Nancy Keller
AOEF007	*(pyroA4); pyrG89*; Δ***nkuA****::argB*; *veA1;* Δ***lacpB***::Af.*pyroA* [c] (pyridoxine prototroph)	This work
AOEF008	*(pyroA4);(pyrG89)* Δ***nkuA****::argB;* Δ***lacpA***::Tr.*pyr4* [b] ; Δ***lacpB***::Af.*pyroA* [c] (pyridoxine & uridine prototroph)	This work
AOEF009 [e]	*(pyroA4);* Δ***nkuA****::argB*; *veA1*; Δ***lacpB***::Af.*pyroA* [c] (pyridoxine & uridine prototroph)	This work
AOEF010 [e]	*(pyroA4);* Δ***lacpB***::Af.*pyroA* [c]*; wA3; riboB2; veA1* (pyridoxine & uridine prototroph)	This work
AOEF011.1	*(pyroA4);* Δ*lacpB::* Af.*pyroA* [c]**;** *veA1;* **(***riboB2*); Af.*riboB* [d]; *wA3*; ***lacpB****$^{1+}$* (pyridoxine, uridine & riboflavin prototroph)	This work
AOEF011.9	*(pyroA4);* Δ*lacpB::* Af.*pyroA* [c]**;** *veA1;* **(***riboB2*); Af.*riboB* [d]; *wA3*; ***lacpB****$^{2+}$* (pyridoxine, uridine & riboflavin prototroph)	This work
AOEF011.7	*(pyroA4);* Δ*lacpB::* Af.*pyroA* [c]*; veA1; (riboB2);* Af.*riboB* [d]*; wA3;* ***lacpB****$^{5+}$* (pyridoxine, uridine & riboflavin prototroph)	This work
AOEF012 [f]	*(pyroA4);(pyrG89);* Δ***lacpA***::Tr.*pyr4* [b] ; Δ***lacpB***::Af.*pyroA* [c] (pyridoxine & uridine prototroph)	This work

Footnote to Supplementary Table 1:

The references given refer to the mutations relevant to this work; other markers are in standard use (cf. Clutterbuck, A.J., 1993. *Aspergillus nidulans*, nuclear genes. In: O'Brien, S. J. (Ed.), Genetic Maps: Locus Maps of Complex Genomes, Vol. 3. Cold Spring Harbor Laboratory Press, New York, pp. 3.71–3.84.).

Abbreviations used: [a] wild-type reference for lactose utilization; [b] *T. reesei* orotidine-5-phosphate decarboxylase (*pyr4*) gene; [c] *A. fumigatus* pyridoxine biosynthesis (*pyroA*) gene; [d] *A. fumigatus* GTP cyclohydrolase (*riboB*) gene; [e] Offspring of a cross between AOEF007 and RJMP155.55; [f] Offspring of a cross between AOEF008 and RJMP155.55.

16 **Supplementary Table S2:** Oligonucleotides used in this study
17

Gene ID	Oligonucleotide sequence (5'-)	Amplicon size, bp	Reference	Application
AN3199 (*lacpA*)	lacpA-Fw: 5'-TTTTTGAATTCGCTGGCTGATGG lacpA-Rv: 5'-TTTTTGAATTCGTGCGGTCTTTGG	796	Fekete *et al.*, 2012	Northern blot
AN2814 (*lacpB*)	lacpB-Fw: 5'-CGTCGGTCGTTTCATTCTCGG lacpB-Rv: 5'- CCGACAGAGCGAGACCCTT	1.063	This study	Northern blot
AN2814 (*lacpB*)	lacpB_copy_Fw: 5'-TTCCTCCTTTCCCACAACGG lacpB_copy_Rv: 5'-ATCCGCAAACCGACACAGAAC	1.550	This study	Southern blot
AN2814 (*lacpB*)	lacpB_RT_Fw: 5'- TCTGTCGTCACCTCGCTCTA lacpB_RT_Rv: 5'- GGAAGAGGTAGTAGAAGGCG	969	This study	RT-PCR
AN2814 (*lacpB*)	AN2814_seq1_Fw: 5'-CCCTCACGCCAACGACTATC AN2814_seq1_Rv: 5'-ACGATAACTGAGGCGAAGCAC	1.015	This study	Sequencing of *lacpB*
AN2814 (*lacpB*)	AN2814_seq2_Fw: 5'-GTGCTTCGCCTCAGTTATCGT AN2814_seq2_Rv: 5'-CGCTTCTTGGACGCCTTGAC	1.081	This study	Sequencing of *lacpB*
AN2814 (*lacpB*)	lacpB_5flank_Fw: 5'-TTCGTTCGGGTCAGCAGCA lacpB5_5flank_Rv: 5'-ACCATAGTAATCCAGCATCGTCGTGCTCGTGGTCAGATG	748	This study	5' flanking arm of *lacpB*
AN2814 (*lacpB*)	lacpB_3flank_fw: 5'-TTCATTATGTAGACACTCGCTCGTTTTTGCCTTGGTTCC lacpB_3flank_rv: 5'-CTGTGATGTGACCTGGAAGC	859	This study	3' flanking arm of *lacpB*
AN2814 (*lacpB*)	Nested_AN2814-Fw: TTCGCCATCAATCCCTT Nested_AN2814-Rv: TCTTAGGTTTGGGGTTGATT	3.467	This study	Complete deletion casette of *lacpB*
AN2814 (*lacpB*)	reintro-lacpB_Fw: 5'-GCTTGTGTTTGAGGAGAT reintro-lacpB_Rv: 5'-AATGCTGTGATGTGACCT	3.717	This study	*lacpB* re-introduction in gene deleted background
AN1577	AN1577-Fw: 5'- ATCGTTGGTTCGTTCTTTGC AN1577-Rv: 5'- ATAGAAGGAGGCGATGTTGA	1.073	This study	RT-PCR
AN6831	AN6831-Fw: 5'- GCCAATACCATCTTCCTCG AN6831-Rv:5'- TGAAGAAGGAGAAGGCAGC	1.047	This study	RT-PCR
AN3200	AN3200-Fw: 5'-CGAGGACCCGCTATTCTACTATTG AN3200-Rv: 5'-CCCTCGCCTCATCCATAAGC	880	Fekete *et al.*, 2012	RT-PCR
pyroA (A. fumigatus)	pyroA_AFU-Fw: GATGCTGGATTACTATGGT pyroA_AFU-Rv: CGAGTGTCTACATATTGAA	1.727	This study	marker gene of *lacpB* deletion casette

Legends to the Supplementary Figures:

Figure S1. Unrooted maximum likelihood tree showing the structural interrelationships of 92 LacpA orthologs and -paralogs encoded by eight genes in 31 species of *Aspergillus*. *A. zonatus* is the most divergent fungus sampled. The paralog proteins are tagged _1 to _8 based on the intron-exon structure of the encoding genes: proteins_1 are the orthologs of *A. nidulans* lactose permease LacpA (at 12 o'clock) in other Aspergilli. For clarity, the four *A. nidulans* proteins in this phylogeny were additionally tagged with their specifying genome locus. Clades of proteins from *A. nidulans*, *A. sydowii*, *Aspergillus* sp. Z5, *A. versicolor* and *A. ustus* – five sequenced fungi within the subgenus *Nidulantes* – are highlighted by the grey background. Branch support statistics are provided by approximate likelihood ratio tests.

Figure S2. Time-profile of growth (open symbols) as well as residual lactose concentration (filled symbols) in batch fermentations of *A. nidulans*. Wild-type strain and *lacpB* mutant at 50 g/L (circles and squares, respectively) and 100 g/L (triangles and diamonds, respectively) initial lactose concentrations. Media were inoculated with conidiospores.

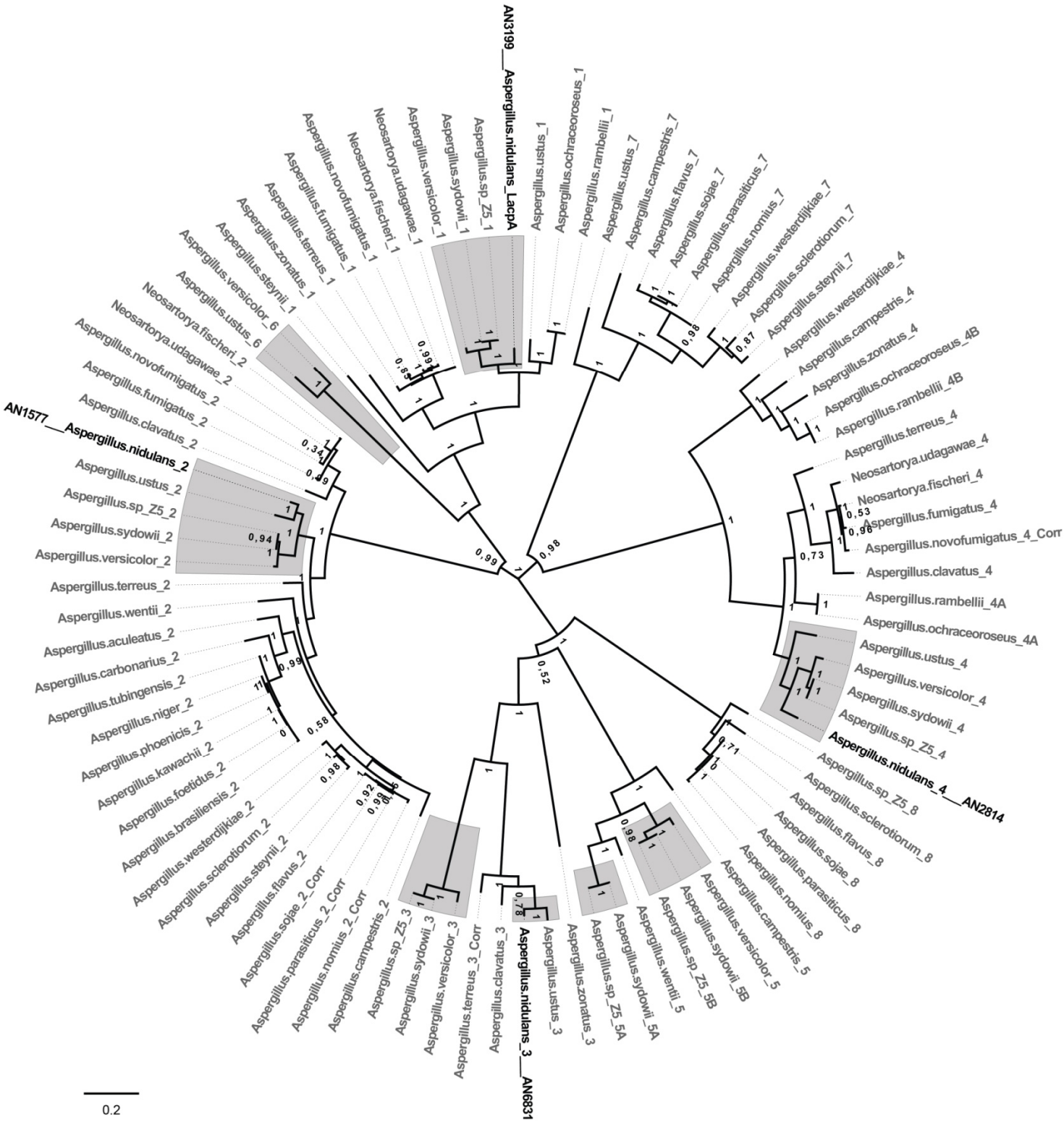
AN3199___Aspergillus.nidulans_LacpA
Aspergillus.ustus_1
Aspergillus.ochraceoroseus_1
Aspergillus.rambellii_1
Aspergillus.ustus_7
Aspergillus.campestris_7
Aspergillus.flavus_7
Aspergillus.sojae_7
Aspergillus.parasiticus_7
Aspergillus.nomius_7
Aspergillus.westerdijkiae_7
Aspergillus.sclerotiorum_7
Aspergillus.steynii_7
Aspergillus.westerdijkiae_4
Aspergillus.campestris_4
Aspergillus.zonatus_4
Aspergillus.ochraceoroseus_4B
Aspergillus.rambellii_4B
Aspergillus.terreus_4
Neosartorya.udagawae_4
Neosartorya.fischeri_4
Aspergillus.fumigatus_4
Aspergillus.novofumigatus_4_Corr
Aspergillus.clavatus_4
Aspergillus.rambellii_4A
Aspergillus.ochraceoroseus_4A
Aspergillus.ustus_4
Aspergillus.versicolor_4
Aspergillus.sydowii_4
Aspergillus.sp_Z5_4
Aspergillus.nidulans_4___AN2814
Aspergillus.sp_Z5_8
Aspergillus.sclerotiorum_8
Aspergillus.flavus_8
Aspergillus.sojae_8
Aspergillus.parasiticus_8
Aspergillus.nomius_8
Aspergillus.campestris_5
Aspergillus.versicolor_5
Aspergillus.sydowii_5B
Aspergillus.sp_Z5_5B
Aspergillus.wentii_5
Aspergillus.sydowii_5A
Aspergillus.sp_Z5_5A
Aspergillus.zonatus_3
Aspergillus.ustus_3
Aspergillus.nidulans_3___AN6831
Aspergillus.clavatus_3
Aspergillus.terreus_3_Corr
Aspergillus.versicolor_3
Aspergillus.sydowii_3
Aspergillus.sp_Z5_3
Aspergillus.campestris_2
Aspergillus.nomius_2_Corr
Aspergillus.parasiticus_2_Corr
Aspergillus.sojae_2_Corr
Aspergillus.flavus_2
Aspergillus.steynii_2
Aspergillus.sclerotiorum_2
Aspergillus.westerdijkiae_2
Aspergillus.brasiliensis_2
Aspergillus.foetidus_2
Aspergillus.kawachii_2
Aspergillus.phoenicis_2
Aspergillus.niger_2
Aspergillus.tubingensis_2
Aspergillus.carbonarius_2
Aspergillus.aculeatus_2
Aspergillus.wentii_2
Aspergillus.terreus_2
Aspergillus.versicolor_2
Aspergillus.sydowii_2
Aspergillus.sp_Z5_2
Aspergillus.ustus_2
AN1577___Aspergillus.nidulans_2
Aspergillus.clavatus_2
Aspergillus.fumigatus_2
Aspergillus.novofumigatus_2
Neosartorya.udagawae_2
Neosartorya.fischeri_2
Aspergillus.ustus_6
Aspergillus.versicolor_6
Aspergillus.steynii_1
Aspergillus.zonatus_1
Aspergillus.terreus_1
Aspergillus.fumigatus_1
Aspergillus.novofumigatus_1
Neosartorya.fischeri_1
Neosartorya.udagawae_1
Aspergillus.versicolor_1
Aspergillus.sydowii_1
Aspergillus.sp_Z5_1
0.2

Figure S1.

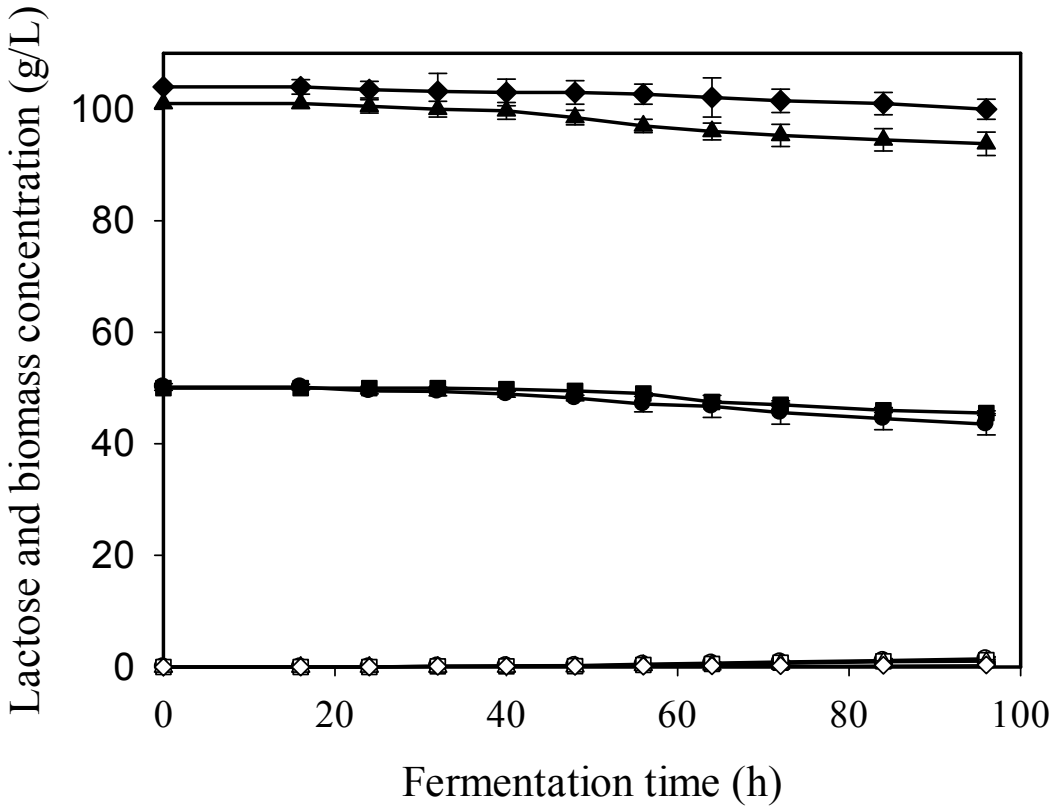
Lactose and biomass concentration (g/L)
0
20
40
60
80
100
0
20
40
60
80
100
Fermentation time (h)

Figure S2.