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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