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Overexpression of a novel endogenous NADH kinase in *Aspergillus nidulans* enhances growth

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ABSTRACT

The complete genome sequence of the filamentous fungi *Aspergillus nidulans* has paved the way for fundamental research on this industrially important species. To the best of our knowledge, this is the first time a gene encoding for ATP-dependent NADH kinase (ATP:NADH 2′-phosphotransferase, EC 2.7.1.86) has been identified. The enzyme has a predicted molecular weight of 49 kDa. We characterised the role of this NADH kinase by genomic integration of the putative gene AN8837.2 under a strong constitutive promoter.

The physiological effects of overexpressed NADH kinase in combination with different aeration rates were studied in well-controlled glucose batch fermentations. Metabolite profiling and metabolic network analysis with [1-^{13}C] glucose were used for characterisation of the strains, and the results demonstrated that NADH kinase activity has paramount influence on growth physiology. Biomass yield on glucose and the maximum specific growth rate increased from 0.47 g/g and 0.22 h^{-1} (wild type) to 0.54 g/g and 0.26 h^{-1} (NADH kinase overexpressed), respectively. The results suggest that overexpression of NADH kinase improves the growth efficiency of the cell by increasing the access to NADPH. Our findings indicate that *A. nidulans* is not optimised for growth in nutrient-rich conditions typically found in laboratory and industrial fermentors. This conclusion may impact the design of new strains capable of generating reducing power in the form of NADPH, which is crucial for efficient production of many industrially important metabolites and enzymes.

1. Introduction

The filamentous fungus *Aspergillus nidulans* does not only belong to a group of industrially important fungi, it also serves as an important model organism for studies on fundamental eukaryotic cell biology. Recent advances in high-throughput experimental tools and the completion of the *A. nidulans* genome sequencing project[2] (Galagan et al., 2005) will accelerate research and facilitate a more systematic use of this fungus as a model organism. A caveat of high-throughput experimental tools is that it can be difficult to analyse and interpret hundreds or thousands of data points, and bring the results into a meaningful physiological context. However, an attractive starting point for physiological studies with high-throughput experimental tools is to alter the expression level of genes encoding enzymes (Jewett et al., 2006).

Metabolism consists of several hundreds enzymatically catalysed reactions of which a large fraction is involved in the conversion of a carbon source into energy via ATP and formation of precursors needed for synthesis of macromolecules. The reactions in the metabolism are highly interconnected, not only due to the common usage of ATP but also through the vital cofactors NADH and NADPH. It is therefore expected that changes in reaction rates where these three cofactors are substrates or products, can have paramount influence on the magnitude of several fluxes and metabolic pools in the cell.

The cofactor NADPH is needed in large quantities for biosynthesis. In the cytosol, NADPH is provided primarily by enzymes in the pentose phosphate (PP) pathway, including glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which each catalyse the reduction of $NADP^+$. The flux through the PP pathway has been reported to increase for high NADPH concentrations in different eukaryotes (Henriksen et al., 1996; Obanye et al., 1996; Pedersen et al., 1999) and to decrease when the need for NADPH production is low (dos Santos et al., 2003). Malic enzyme catalyses the reaction

$$\text{L-malate} + NADP^+ \rightarrow \text{pyruvate} + CO_2 + NADPH$$

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[2] http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/

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and has been shown to be a major source of cytosolic NADPH for lipid synthesis in *A. nidulans* (Wynn and Ratledge, 1997) and other filamentous fungi (Wynn et al., 1999). There is also a need for mitochondrial NADPH since it cannot be transported from the cytosol across the mitochondrial membrane. The sources of NADPH in the mitochondrion are less clear. In mammalian cells, mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (NADPH-IDHm) has been reported to be an important source of NADPH. However, deletion of the corresponding gene in baker's yeast *Saccharomyces cerevisiae* (IDP1) had no effects on cell growth or oxidative stress sensitivity (Minard et al., 1998). Based on these observations it is clear that NADPH-IDHm cannot be the only means by which NADPH is produced in eukaryotic mitochondria.

Catabolic reactions primarily generate reducing power in the form of NADH rather than NADPH. The NAD^+/H kinases are the only known enzymes that convert NAD^+/H to $NADP^+$/H in a single reaction step (Raffaelli et al., 2004, Mori et al., 2005a):

$$ATP + NAD^+/H \rightarrow ADP + NADP^+/H$$

Loss of NAD^+/H kinase activity in the cell is predicted to have pleiotropic effects. Most multicellular eukaryotic organisms only have one NAD^+/H kinase gene. All of the NAD^+/H kinases are highly conserved from simple bacteria such as *Escherichia coli* to mammals. The human enzyme preferentially converts NAD^+ to $NADP^+$ (Lerner et al., 2001). In contrast, it has been shown by biochemical methods that the yeast *S. cerevisiae* has three NAD^+/H kinase genes, *UTR1*, *YEF1*, *POS5* (Outten and Culotta, 2003; Shi et al., 2005; Strand et al., 2003).

Despite academic and industrial importance, the community of researchers involved in the study of *A. nidulans* is far smaller than for the popular model organisms *E. coli* and *S. cerevisiae*. Consequently, several fundamental questions have not yet been addressed, and it is therefore crucial for our understanding of *A. nidulans*' physiology that the important interplay between the redox cofactors NADPH and NADH is elucidated.

In this study the gene for an ATP-dependent NADH kinase (ATP:NADH 2′-phosphotransferase, EC 2.7.1.86), which to date only has been described in yeast and plants, was identified in the fungus *A. nidulans*. NADH kinase is expected to have a large impact on the physiology, and consequently it is not obvious what the phenotype of a highly active NADH kinase would be in a higher eukaryote grown in controlled fermentors. Here we sought to investigate the phenotypic effect of overexpressing the gene encoding NADH kinase in *A. nidulans* by using metabolic network analysis and metabolite profiling. With these experimental tools it is possible to quantify and analyse the interplay between fluxes and metabolic pools in the central carbon metabolism of *A. nidulans* after genetic perturbations.

2. Materials and methods

2.1. Microorganism and culture conditions

A defined medium containing trace metal elements was used for all *A. nidulans* cultivations. The media used had the following composition (per litre): 15 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, 2 g NaCl, 0.2 g $CaCl_2$, 0.7 g arginine and 1 ml/l trace element solution. Trace element solution composition (per litre): 14.3 g $ZnSO_4 \cdot 7H_2O$, 13.8 g $FeSO_4 \cdot 7H_2O$ and 2.5 g $CuSO_4 \cdot 5H_2O$. The carbon sources used were glucose, xylose or acetate in a concentration of 10 g/l. Batch cultivations were carried out in laboratory fermenters and baffled Erlenmeyer flasks. The physiological characterisation of the strains was performed at 30 °C in well-controlled 1.5 l fermenters (working volume of 1.2 l) equipped with two disc-turbine impellers. The stirring rate was 350 rpm and the pH was kept constant at 5.5 by addition of 2 M NaOH or HCl. The fermentors were sparged with air at a constant flow rate of 0.1 vvm (termed "oxygen limitation") or 1.0 vvm (termed "fully aerated") resulting in a dissolved oxygen tension of at least 17% of saturation.

The NADH kinase enzyme activity measurements for different carbon sources were based on duplicate cultivations in 250 ml Erlenmeyer flasks (100 ml medium). Cells were incubated at 30 °C in an orbital shaker (200 rpm).

2.2. Strain construction and analytical methods

A putative NADH kinase gene AN8837.2 (subsequently called *ndkA*) (GI: 49147770) from *A. nidulans*, was amplified using the Expand High Fidelity PCR System (Roche, Switzerland) with primers AN8837.2_FseI_fw (5′-ATCAggccggccATGCTGTCAACGA-TAAAATTG-3′) and AN8837.2_AscI_re (5′-GATggcgcgccTCACTCTC-CAAAGGGGTAG-3′) containing the start and stop codons as well as the restriction sites FseI and AscI.

The obtained PCR product of the expected length was then cloned into the available unique restriction sites, *Fse*I and *Asc*I in the pTr04 expression vector, resulting in pTr04AN8837.2 which was verified by sequencing. The pTr04 vector has been derived from pBARGPE1 (Brunelli and Pall, 1993) which contains the *gpdA* promoter from *A. nidulans*, linked to a multiple cloning site (MCS) and the TrpC terminator.

A. nidulans AR1 [*pyrG*89 *argB2*; *veA1*] was transformed with pTr04AN8837.2 as described in Nielsen et al. (2006), *pyrG* from *A. fumigatus* was used as the selection marker, and selection yielded the transformant *A. nidulans* gp*ndk*74.

The *A. nidulans* strain A4 is a natural isolate and therefore has no explicit genotype, and the strain AR1 is directly derived from it.

Fermentation samples were immediately filtered and stored at −20 °C until time of analysis. Medium concentrations of glucose, xylose, glycerol, acetate, succinate and pyruvate were determined by HPLC analysis using an Aminex HPX-87H column (BioRad). The column was kept at 45 °C and eluted at 0.6 ml/min with 5 mM H_2SO_4. A Waters (410) refractive index detector was used together with a UV detector for compound detection.

NADH kinase activity was measured spectrophotometrically at 340 nm in a reaction mixture as described before (Turner et al., 2005). Protein concentrations were determined by the Bradford method (Bradford, 1976).

2.3. Sampling, extraction and determination of intracellular intermediary metabolites

Samples for analysis of intracellular metabolites were taken in triplicates at the mid-exponential growth phase. Ten millilitres fermentation broth was immediately quenched in 20 ml cold (−40 °C) 72% methanol. After quenching, the cells were separated from the quenched solution by centrifugation at 10,000*g* for 20 min at −20 °C and the intracellular metabolites were extracted as described by Villas-Boas et al. (2005). Finally, the extracted samples were lyophilised and stored at −80 °C until further analysis. Variation of intracellular metabolite levels across replicate samples did not exceed 15%. Organic acids (including amino acids) were analysed by gas chromatography–mass spectrometry (GC–MS). The GC–MS analysis was performed on an Agilent HP 6890 series GC chromatograph (Palo Alto, CA, USA) coupled to a HP 5973 quadrupole MSD (EI) operated at 70 eV. The GC was equipped with a 4.0 mm i.d. Siltek gooseneck split less deactivated liner (Restek, Bellefonte, PA, USA), and Supelco (Bellefonte, PA, USA) SLB-5 MS column (15 m, 0.25 mm i.d., 0.25 μm film thickness). The profile of identified intracellular