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An accurate normalization strategy for RT-qPCR in *Hypocrea jecorina* (*Trichoderma reesei*)

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ABSTRACT

Hypocrea jecorina is an important, filamentous fungus due to its effective production of hydrolytic enzymes. Gene expression studies provide deeper insight into environment sensing and cellular response mechanisms. Reverse transcription-quantitative PCR is a gene-specific and powerful tool to measure even minor changes in mRNA composition. An accurate normalization strategy is absolutely necessary for appropriate interpretation of reverse transcription-quantitative PCR results. One frequently applied strategy is the usage of a reference gene. Adequate reference genes for *Hypocrea* have not been published so far. By using the NormFinder and geNorm softwares, we evaluated the most stable genes amongst six potential reference genes in 34 samples from diverse cultivation conditions. Under those experimental conditions, *sar1* encoding for a small GTPase was found to be the most stable gene, whereas *act* encoding for actin was not amongst the best validated ones. The influence of the reference system on the expression data is demonstrated by analysis of two target genes, encoding for the Xylanase regulator 1 and for Xylanase II. We further validated obtained xylanase 2 transcription rates with the corresponding enzyme activity.

1. Introduction

Hypocrea jecorina (anamorph: *Trichoderma reesei* (Kuhls et al., 1996)) is a filamentous ascomycete widely used because of its high secretory capacity for hydrolyzing enzymes. Hydrolases secreted by this fungus are used in a broad range of industrial applications covering, for instance pulp and paper industry (Buchert et al., 1998; Welt and Dinus, 1995; Noé et al., 1986), food and feed industry (Galante et al., 1993; Walsh et al., 1993; Lanzarini and Pifferi, 1989), and textile industries (Koo et al., 1994; Kumar et al., 1994; Pedersen et al., 1992) as well as biofuels and bioenergy (Himmel et al., 2007; Hahn-Hägerdal et al., 2006; Ragauskas et al., 2006).

Detailed information on the regulatory mechanisms of hydrolase expression in *Hypocrea*, will enable the systematically engineering of existing production strains in order to increase their expression efficiency. Transcription analysis is the basis for the understanding of regulatory mechanisms governing gene expression. One powerful tool to measure the mRNA content is reverse transcription-quantitative PCR (RT-qPCR). Major advantages of this method are its high sensitivity, large dynamic range, and accurate quantification (Huggett et al., 2005). However, an accurate and robust normalization system is needed when performing relative quantification of qPCR data. Normalizing to a stably expressed gene of the target organism, often called reference or housekeeping gene, is a powerful method for internal error prevention. In general, when applying mRNA quantification techniques, an error is caused by the multistage process required to extract, process (i.e. *in vitro* reverse transcription) and detect mRNA.

A major challenge is to find a suitable reference gene. Even genes like β-actin, believed to be stable, turned out to be inapplicable under certain conditions (e.g. comparing different mouse tissues) (Barbu and Dautry, 1989). In *Hypocrea*, as in other filamentous fungi, only a single gene encoding for actin is described (Matheucci et al., 1995). So far, only less is known about its suitability as a reference gene in fungi. A study performed in *Saccharomyces* reveals that actin scores at the third best position when tested with NormFinder and geNorm (Stahlberg et al., 2008). In *Aspergillus niger* an investigation done by means of geNorm demonstrated that under the tested conditions the actin encoding gene belongs to the most stable ones (Bohle et al., 2007). An important requirement for such a reference gene is its robust expression under all conditions applied in a certain experiment. Therefore, a careful evaluation is necessary to obtain one or even more suitable genes for normalization. Vandesompele and co-workers suggest the use of multiple reference genes rather than relying on a single one (Vandesompele et al., 2002). However, it depends on the experimental setup how many reference genes have to be included in order to obtain consistent and reliable results. Especially for the

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setup of new experimental conditions, it has to be evaluated if a certain set of reference genes is suitable or not. Different approaches have been published, which allow the evaluation of multiple reference genes (Andersen et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002). The software geNorm ranks the genes according to the similarity of their expression profiles by a pairwise comparison (Vandesompele et al., 2002). The authors calculated the average pairwise variation of a particular gene with all other control genes and denominated it M, the internal control gene-stability measure. Within this system, genes with the lowest *M* values have the most stable expression. Another calculation method is used by the program NormFinder (Andersen et al., 2004), which uses a model-based approach for the estimation of expression variation. The advantage of this algorithm compared to geNorm is that it can deal with systematic differences in the data set like different tissues or strains.

The relative expression software tool (REST; version: 2008) developed by Pfaffl and co-workers allows the estimation of gene expression using qPCR amplification data (i.e. the threshold cycle values). This software enables the measurement of uncertainty in expression ratios by introducing randomization and bootstrapping techniques. Confidence intervals for expression levels allow measurement of not only the statistical significance of deviations but also of their likely magnitude even in the presence of outliers (Pfaffl et al., 2002).

In this study we analyzed six potential reference genes with geNorm and NormFinder in order to find suitable normalization conditions for *H. jecorina*. With REST software we calculated the expression profiles of two target genes (*xyn2*, *xyr1*) applying different combinations of reference systems. *xyr1* encodes for the Xylanase regulator 1 (Xyr1), which is the main activator of hydrolase expression in *H. jecorina* (Stricker et al., 2006, 2007). Xyr1 itself is regulated at the transcriptional level by a repression/derepression mechanism. The constitutive expression of *xyr1* leads to a significant elevation/deregulation of hydrolytic enzymes encoding genes transcription in comparison to what is observed in the parental strain (Mach-Aigner et al., 2008). *xyn2* encodes for Xylanase II (XYNII) and this enzyme is responsible for >50% of xylanolytic activity in the culture filtrate of *H. jecorina* grown on xylan (Törrönen et al., 1992, 1993, 1994). In this study, we illustrate the normalization strategy choice impact upon the relative gene expression output. Examples of results obtained by using stable and unstable reference genes for transcript analysis are highlighted. Furthermore, we evaluated the normalization system by a correlation analysis of transcription rates with enzyme activity.

2. Materials and methods

2.1. Strains

The following *H. jecorina* (*T. reesei*) strains were used throughout this study: QM9414 (ATCC 26921, a cellulase hyperproducing mutant derived from wild-type strain QM6a (Mäntylä et al., 1992)), nx7 strain (a QM9414 recombinant strain constitutively expressing *xyr1* under the *nag1* promoter control) (Mach-Aigner et al., 2008), Δ*ace2* strain (a QM9414 *ace2* deletion strain) and Re*ace2* strain (an *ace2* retransformation strain of the Δ*ace2* strain) (Stricker et al., 2008). All strains were maintained on malt agar.

2.2. Growth conditions

For replacement experiments mycelia of the strains QM9414 and nx7 were pre-cultured in 1-liter Erlenmeyer flasks on a rotary shaker at 30 °C for 18 h in 250 mL of Mandels–Andreotti (MA) medium (Mandels, 1985) containing 1% (w/v) glycerol as the sole carbon source. Conidia (final concentration, 10^8 per liter) were used as the inoculum. Pre-grown mycelia were washed and thereafter equal amounts were resuspended in 50 mL MA medium containing 1% (w/v) glucose or D-xylose, or 1.5 mM xylobiose, or a medium without a carbon source. Incubation was continued at 30 °C and shaking (250 rpm). 15-mL samples were taken after 3, 5, and 8 h (Mach-Aigner et al., 2008).

Cultivation of Δ*ace2* and Re*ace2* strain in a bench top bioreactor (Applikon Biotechnology, Schiedam, Netherlands) was carried out using 1 L medium adjusted to pH 4.5 comprising 20 g beech wood xylan (Lenzing AG, Lenzing, Austria), 2.8 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7H_2O$, 4 g KH_2PO_4, 0.5 g NaCl, 0.5 g Tween 80, 0.1 g peptone, 5 mg $FeSO_4 \cdot 7H_2O$, 1.7 mg $MnSO_4 \cdot H_2O$, 1.4 mg $ZnSO_4 \cdot 7H_2O$, and 2 mg $CaCl_2 \cdot 2H_2O$ in distilled water. Some drops glanapon (Becker, Vienna, Austria) were added to the medium to avoid excessive foam formation. For 1 L, 10^8 conidia were used as inoculum. Cultivation was performed at 30 °C, pH 4.5, 0.3 l^{-1} min^{-1} aeration rate and 500 rpm agitation rate. Timing of sample drawing was planned according to the expected time points of *xyn2* transcript formation. Each sample drawing was followed by a microscopic analysis for infection control. Culture supernatant and mycelia were separated by filtration through GF/F glass microfiber filters (Whatman, Brentford, UK). Computer-aided process control and monitoring was performed using the LIME Process Control software (ATS, Vienna, Austria) (Stricker et al., 2008).

All mycelia samples were harvested using a textile filter (Miracloth, Calbiochem, USA), washed and flash-frozen in liquid nitrogen.

2.3. Xylanase enzyme assay

Endo-xylanase activity was measured applying Xylazyme AX Tablets (Megazyme, Wicklow, Ireland) according to the manufacturer's instructions at a pH of 4.7 which is within the range of the pH optimum of Xylanase II (pH optimum 4.5–5.5) but not of Xylanase I (pH optimum 3.5–4.0) (Tenkanen et al., 1992). One Unit of activity is defined as the amount of enzyme required to release one micromole of xylose reducing-sugar-equivalents per minute under the defined assay conditions (Stricker et al., 2008).

2.4. RNA-extraction and reverse transcription

Harvested mycelia were homogenized in 1 mL peqGOLD TriFast DNA/RNA/protein purification system (PEQLAB Biotechnologie, Erlangen, Germany) using a FastPrep FP120 BIO101 ThermoSavant cell disrupter (Qbiogene, Carlsbad, USA). DNA and RNA were simultaneously isolated in a two-step-process according to the manufacturer's instructions. Total RNA quantity was determined by means of UV-VIS-spectrophotometry. Quality of extracted RNA was determined as the 260 nm/280 nm ratio. Samples with a ratio between 1.8 and 2.2 were further processed immediately (Pfaffl et al., 2004). Synthesis of cDNA from mRNA was carried out applying RevertAidTM H Minus First Strand cDNA synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions. 0.5 μg of each mRNA sample was used for reverse transcription. cDNA samples were diluted 1:100 prior to qPCR.

2.5. Selection of reference genes and primer design

Candidate reference genes were selected according to previously published reference gene analysis performed in *Aspergillus niger* (Bohle et al., 2007) and *Hypocrea atroviride* (Pucher, 2006). Corresponding genes in *H. jecorina* were found by means of a tblastn analysis (standard settings) using the published genome sequence