APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Six novel constitutive promoters for metabolic engineering of *Aspergillus niger*

Marzena Blumhoff · Matthias G. Steiger · Hans Marx ·
Diethard Mattanovich · Michael Sauer

Abstract Genetic tools for the fine-tuning of gene expression levels are a prerequisite for rational strain optimization through metabolic engineering. While *Aspergillus niger* is an industrially important fungus, widely used for production of organic acids and heterologous proteins, the available genetic tool box for this organism is still rather limited. Here, we characterize six novel constitutive promoters of *A. niger* providing different expression levels. The selection of the promoters was based on published transcription data of *A. niger*. The promoter strength was determined with the β-glucuronidase (*gusA*) reporter gene of *Escherichia coli*. The six promoters covered a GUS activity range of two to three orders of magnitude depending on the strain background. In order to demonstrate the power of the newly characterized promoters for metabolic engineering, they were used for heterologous expression of the *cis*-aconitate decarboxylase (*cad1*) gene of *Aspergillus terreus*, allowing the production of the building block chemical itaconic acid with *A. niger*. The CAD activity, dependent on the choice of promoter, showed a positive correlation with the specific productivity of itaconic acid. Product titers from the detection limit to up to 570 mg/L proved that the set of constitutive promoters is a powerful tool for the fine-tuning of metabolic pathways for the improvement of industrial production processes.

Introduction

Aspergillus niger is a widely used host organism for the industrial production of food processing enzymes and metabolites such as organic acids or antibiotics (Sauer et al. 2008; Tevz et al. 2010; Dashtban et al. 2011; Frisvad et al. 2011). Rational strain design becomes increasingly important for this microbial cell factory. To this end analysis and modelling of the cellular metabolism is followed by design of the metabolic pathways using genetic engineering (Andersen and Nielsen 2009; Kim et al. 2012; Sauer and Mattanovich 2012). A prerequisite for such rational engineering approaches is an adequate genetic tool box including markers, vectors, and promoters among others. The set of promoters, which have been described for heterologous gene expression in *A. niger* is currently quite focused on promoters providing a high expression level of the target gene (Fleissner and Dersch 2010). For fine-tuning of the gene expression, some promoters have been described which are inducible, thereby allowing for low and high expression from the same construct. However, these

M. Blumhoff · M. Sauer
School of Bioengineering,
University of Applied Sciences FH-Campus Wien,
Muthgasse 56,
1190 Vienna, Austria

M. Blumhoff · H. Marx · D. Mattanovich · M. Sauer
Department of Biotechnology, BOKU-VIBT University of Natural Resources and Life Sciences,
Muthgasse 18,
1190 Vienna, Austria

M. G. Steiger (✉) · D. Mattanovich · M. Sauer
Austrian Centre of Industrial Biotechnology (ACIB GmbH),
Muthgasse 11,
1190 Vienna, Austria
e-mail: matthias.steiger@acib.at

promoters require usually strictly defined conditions for high or low gene expression, respectively. Many of them are dependent on the carbon or nitrogen source in the culture medium such as the glucoamylase promoter P*glaA* of *A. niger* (Ganzlin and Rinas 2008), the alcohol dehydrogenase promoter P*alcA* of *Aspergillus nidulans* (Waring et al. 1989), or the Taka-amylase A promoter P*amyB* of *Aspergillus oryzae* (Kanemori et al. 1999). Other promoters are induced only in the presence of certain substances, like thiamine (thiamine promoter P*thiA* of *A. oryzae*) (Shoji et al. 2005) or estrogen (human estrogen receptor hERα) (Pachlinger et al. 2005). Recently, Meyer et al. (2011) have described a very tight, tuneable Tet-on (induced by tetracycline) expression system for *A. niger*, that is dependent on the doxycycline concentration. The advantage of these systems is the possible fine regulation of gene expression depending on the concentration of the inducer, which is very useful for scientific purposes. However, in an industrial production context the dependence on the lack or addition of a specific compound or specific other medium constraints are a disadvantage. Construction of a metabolic network, which constitutively gives rise to the required combination of enzyme activities allows for flexibility for bioprocess design and is not dependent on special media (components) and is therefore the preferred solution.

Among the constitutive promoters, which have been described for filamentous fungi are the alcohol dehydrogenase promoter P*adhA* of *A. niger*, the pyruvate kinase promoter P*pki* of *Trichoderma reesei* (Limón et al. 1999) or the glyceraldehyde-3-phosphate dehydrogenase promoter P*gpdA* of *A. nidulans* (Hunter et al. 1992). While P*gpdA* is by far the most frequently used promoter for genetic engineering of *Aspergillus* ssp. all of them are useful for high-level gene expression.

However, the optimization of complex networks of metabolic pathways does not always require the highest activity of all genes of interest. In a lot of cases, it is more a question of fine-tuning and balancing of the involved activities. Notwithstanding, constitutive promoters providing different levels of expression strength are hardly characterized and not available with a sufficient degree of variability for filamentous fungi.

In this study, we cloned and characterized six novel constitutive promoters of the industrially important fungus *A. niger*, providing different levels from low to high expression of the target genes. In order to prove their functionality as a useful tool for metabolic engineering, we applied them on the metabolic pathway leading to the production of itaconic acid. Since *A. niger* lacks cis-aconitate decarboxylase activity, this organism is not naturally able to produce itaconic acid (Kubicek and Karaffa 2006). Heterologous expression of the *cad1* gene of *Aspergillus terreus* in *A. niger* results in strains producing low amounts of itaconic acid in the culture medium (Li et al. 2011). In our study, we transformed the *cad1* coding sequence of *A.*

terreus under the control of six constitutive promoters to *A. niger* and confirmed their functionality as novel tool for metabolic engineering.

Materials and methods

Strains and media

Escherichia coli TOP10 was used as host for recombinant DNA manipulation. *A. niger* CBS 513.88 and ATCC 1015 were used as parental strains for *A. niger* transformation.

E. coli transformants were grown on LB (Sambrook and Russell 2001) supplemented with 75 μg/mL hygromycin. *A. niger* was cultured in liquid ME or solid MEA (3 % malt extract, 0.5 % peptone, and ±1.5 % agar), supplemented with 150 μg/mL hygromycin, or in Vogel's minimal medium (2.5 g/L Na$_3$-citrate $2\times$H$_2$O, 5 g/L KH$_2$PO$_4$, 2 g/L NH$_4$NO$_3$, 0.2 g/L MgSO$_4$ $7\times$H$_2$O, 0.1 g/L CaCl$_2$ $2\times$H$_2$O, 0.5 mg/L citric acid $1\times$H$_2$O, 0.5 mg/L ZnSO$_4$ $7\times$H$_2$O, 0.1 mg/L Fe(NH$_4$)$_2$ (SO$_4$)$_2$ $6\times$H$_2$O, 0.025 mg/L CuSO$_4$ $5\times$H$_2$O, 0.005 mg/L MnSO$_4$ $1\times$H$_2$O, 0.005 mg/L H$_3$BO$_3$, 0.005 mg/L Na$_2$MoO$_4$ $2\times$H$_2$O, and 0.005 mg/L biotin) (Vogel 1956) without MnSO$_4$ and supplemented with 10 % glucose as carbon source. For the osmotic stabilization, 1.2 M sorbitol was added to the medium after transformation. Top agar was composed of osmotically stabilized MEA containing 0.75 % agar. Bottom agar was composed of osmotically stabilized MEA supplemented with 180 μg/mL hygromycin. For the detection of β-glucuronidase activity, additional 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc, 70 mg /L) was added to the plates.

Selection of constitutive promoters of *A. niger* using microarray expression data

The Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) was used to retrieve five gene expression data sets (GSE11405, GSE17329, GSE14285 (van den Berg et al. 2010), GSE17641 (Meyer et al. 2009), and GSE21752 (Jørgensen et al. 2010) of *A. niger* based on the Affymetrix chip platform GPL6758. For each gene, the average signal intensity was determined by calculation of the arithmetic mean from all data sets. Then the genes were grouped into two arbitrary subgroups (A and B): the first (A) containing all highly expressed genes with an average signal intensity >1,000 (463 genes) and a second group (B) containing weaker expressed genes with an average signal intensity between 230 and 299 (500 genes). In both subgroups, the most stably expressed genes were determined using the software NormFinder (Andersen et al. 2004). This software uses a model-based approach for the estimation of

expression variation and can deal with heterogeneous data sets subdivided in distinct groups. With regard to this ability the expression data sets were subdivided into five sample groups according to the five gene expression data sets used for the analysis. The stability value was determined and, out of the first ten genes with the highest stability, three genes of each gene subgroup were randomly selected (Table 1). 1.5 kb fragments located upstream of the coding sequences of the six selected genes listed in Table 1 were PCR amplified from genomic DNA of *A. niger* ATCC 1015 and used as promoter sequences in this study.

Construction of *gusA* and *cad1* expression cassettes

Plasmids were constructed using a previously published enzymatic assembly method (Gibson et al. 2009). Target DNA fragments were obtained by touchdown PCR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) under the following conditions: 98 °C for 10 s, 14 cycles of 98 °C for 10 s, 70 °C minus 1 °C/cycle for 30 s, 72 °C for 90–150 s, 25 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 90–150 s, and a final extension at 72 °C for 10 min. The primers are specified in Table S1 in the Electronic supplementary material (ESM). Two series of plasmids A and B containing (A) *gusA* of *E. coli* (plasmids pGUS_mbfA, pGUS_coxA, pGUS_srpB, pGUS_tvdA, pGUS_mdhA, pGUS_manB) or (B) *cad1* of *A. terreus* (plasmids pCAD_mbfA, pCAD_coxA, pCAD_srpB, pCAD_tvdA, pCAD_mdhA, pCAD_manB) were constructed for homologous integration of the expression cassette into the *mus53* locus. The plasmids consisted of the following 11 fragments (in order): (1) one of the *A. niger* promoters amplified from genomic DNA of strain ATCC 1015 (Table 1), followed by the *gusA* coding sequence (2A) of *E. coli* amplified from genomic DNA of strain TOP10, or the *cad1* coding sequence of *A. terreus* (2B) amplified from genomic DNA of strain DSM 62071 (Kanamasa et al. 2008); (3) the T*trpC* terminator

amplified from pAN52-1Not (GeneBank Accession No. Z32524); (4) the *hph* gene under the P*pki* promoter and the T*cbh2* terminator of *Hypocrea jecorina* amplified from pRLMex30 (Mach et al. 1994) suitable for selection of both, *E. coli* and filamentous fungi (Steiger et al. 2011); (5) the T*trpC* terminator amplified from pAN52-1Not (GeneBank Accession No. Z32524) suitable for selection marker recovery; (6) 1 kb of the 3′ flanking region of *mus53* of *A. niger* amplified from genomic DNA of strain ATCC 1015; (7) *PmeI* restriction site; (8A/8B) AMA1 fungal origin of replication amplified in two pieces from pRG3-AMA1-NotI (Osherov et al. 2000) kindly provided by Joseph Strauss, University of Natural Resources and Life Sciences, Vienna (BOKU); (9) origin of replication for *E. coli* amplified from pUC19 (GenBank Accession No. M77789); (10) *PmeI* restriction site; (11) 1 kb of the 5′ flanking region of *mus53* of *A. niger* amplified from genomic DNA of strain ATCC 1015. The map of the plasmids is included in the online resource (Fig. S1 in the ESM). Two or three overlapping PCR products were fused by secondary PCR. Four secondary fragments were enzymatically assembled for each plasmid. Obtained plasmids were cloned into *E. coli* and isolated according to standard methods (Sambrook and Russell 2001). In order to obtain integrative expression cassettes, the AMA1 and ori for *E. coli* were cut out with the *PmeI* restriction enzyme.

Transformation of *A. niger*

The preparation of protoplasts and the transformation of *A. niger* were performed based on Yelton et al. (1984). Conidia (1×10^8) were cultivated in 250 mL ME for 16–18 h at 30 °C. The mycelium was harvested by filtration through Miracloth (Calbiochem) and washed with deionized water. Protoplastation was achieved in the presence of 5 g/L of lysing enzymes from *Trichoderma harzianum* (Sigma®, L1412) in SMC (1.2 M sorbitol, 50 mM CaCl$_2$, 20 mM Mes/NaOH, pH 5.8),

Table 1 Promoters used in this study

Promoter name	Gene name	Protein ID of *Aspergillus niger* ATCC 1015	Protein ID of *A. niger* CBS 513.88	Annotation
P*mbfA*	*mbfA*	ID37453	An02g12390	Strong similarity to multiprotein bridging factor 1 Mbf1—*Saccharomyces cerevisiae*
P*coxA*	*coxA*	ID199998	An07g07390	Strong similarity to subunit IV of cytochrome c oxidase Cox4—*S. cerevisiae*
P*srpB*	*srpB*	ID55055	An16g08910	Similarity to nucleolar protein Srp40—*S. cerevisiae*
P*tvdA*	*tvdA*	ID203695	An04g01530	Strong similarity to transport vesicle docking protein Pep12—*S. cerevisiae*
P*mdhA*	*mdhA*	ID183145	An15g00070	Strong similarity to malate dehydrogenase precursor MDH—*Mus musculus*
P*manB*	*manB*	ID49344	An14g03520	Strong similarity to filamentous growth protein Dfg5—*S. cerevisiae*, putative alpha-1,6-mannase

Promoter names were chosen according to the annotation by Andersen et al. (2011). Given protein IDs refer also to Andersen et al. (2011)

for 4 h at 37 °C, 120 rpm. The protoplasts were collected by filtration through Miracloth and centrifugation at 2,000×g at 4 °C for 5 min. After a washing step with cold STC (1.2 M sorbitol, 50 mM CaCl$_2$, 10 mM Tris/HCl, pH 7.5), the proto-plasts were resuspended in STC and directly used for transfor-mation. Five micrograms of linear DNA (30 μL) was mixed with 100 μL of at least 10^8 protoplasts and 330 μL freshly prepared PEG solution (25 % PEG 6000, 50 mM CaCl$_2$, 10 mM Tris/HCl, pH 7.5), and incubated for 20 min on ice. After the addition of 2 mL PEG solution and 10 min incu-bation at room temperature, the protoplasts mixture was diluted with 4 mL of STC. The aliquots were mixed with 4 mL of liquid top agar, spread on bottom agar containing the antibiotic and incubated at 30 °C for 4–6 days. All trans-formants were three times purified by single colony isolation on the selection medium (Daud et al. 1985). The correct integration was verified by PCR analysis using specific ge-nomic primers on both sides of the expression cassette. Only clones were further analyzed, which showed positive PCR results for both, the 5′and 3′ end of the expression cassette.

Preparation of cell-free extracts

To obtain cell-free extracts, fungi were cultivated on MEA for 26–28 h at 30 °C (inoculated with conidia). The mycelium was harvested into tubes containing either (for GUS activity) 2× GUS reaction buffer (×1 reaction buffer contains, 50 mM sodium phosphate, pH 7; 1 mM EDTA; 0.001 % Triton X-100; 5 mM DTT) or (for CAD activity) 0.2 M sodium phos-phate buffer (pH 6.2), and disrupted with 0.4–0.6 mm acid washed glass beads (five times, 20 s, 4 m/s). The protein concentration was determined based on Bradford's protocol (Bradford 1976) using the Bio-Rad Protein Assay according to the manufacturer's instructions.

β-glucuronidase activity assay

For the measurement of the β-glucuronidase (GUS) activity, 10 μL of the cell-free extract in eight different dilutions was mixed with 40 μL of ×2 GUS reaction buffer and diluted with 50 μL of 4 mM 4-methylumbelliferyl-β-D-glucuronide (MUG). The reaction was carried out at 37 °C. The fluores-cence was measured at excitation/emission of 355/460 nm with the time interval of 80 s. The linear range of fluores-cence increase was used for the calculation of the GUS activity. One GUS unit is defined as the amount of the enzyme that catalyzes the conversion of 1 μM MUG/min.

Cis-aconitate decarboxylase activity assay

The *cis*-aconitate decarboxylase (CAD) activity was mea-sured according to Bentley and Thiessen (1957). The reac-tion containing 8 mM freshly prepared cis-aconitic acid in 0.2 M sodium phosphate buffer (pH 6.2) and 200 μL cell-free extract, was carried out for 10 min at 37 °C and heat inactivated at 80 °C for 20 min. The CAD activity was measured by direct detection of itaconic acid as a reaction product by HPLC (as described below). One CAD unit is defined as the amount of the enzyme that catalyzes the conversion of 1 μM CAD/min.

Shake flask cultivation of cad1 transformants of A. niger

Conidia (5×10^7 or 5×10^8) were cultivated for 432 h at 33 °C, shaking (120 rpm) in 1 L baffled shake flasks containing 100 mL Vogel's medium. Final concentration of the organic acids produced in the medium and the remaining glucose was determined by HPLC. The biomass was harvested by filtration through Miracloth. The dry weight was determined by drying the biomass in the oven at 110 °C for 2 days.

HPLC measurements

Nine hundred microliters of the HPLC samples were mixed with 100 μL of 0.04 M H$_2$SO$_4$, filtrated through 0.20 μm RC membrane filters and measured by HPLC (Shimadzu) with a Phenomenex Rezex ROA column (300×7.8 mm). A refraction index detector (RID-10A, Shimadzu) was used for detection of glucose, while a PDA detector (SPD-M20A, Shimadzu) at 200 nm was used for itaconic acid. The column was operated at 60 °C temperature, 1 mL/min flow rate, and 0.004 M H$_2$SO$_4$ as mobile phase.

Results

Selection of constitutive promoters of A. niger

A constitutive promoter, unlike an induced promoter, should have a constant transcription level under as many as possi-ble different conditions. In order to find constitutive pro-moters spanning a wide range of expression levels, we reinvestigated all microarray expression data sets which were publicly available at the time being, in order to have a high variability in the cultivation conditions (e.g., different carbon and nitrogen sources, bioreactor and shake flask cultures). These data sets were analyzed for constitutively expressed genes with the software NormFinder. This soft-ware was originally developed to find stably expressed reference genes for quantitative PCR analysis. The underly-ing algorithm calculates which gene shows the lowest var-iance in expression level throughout a data set. The selected genes from this analysis are shown in Table 1.
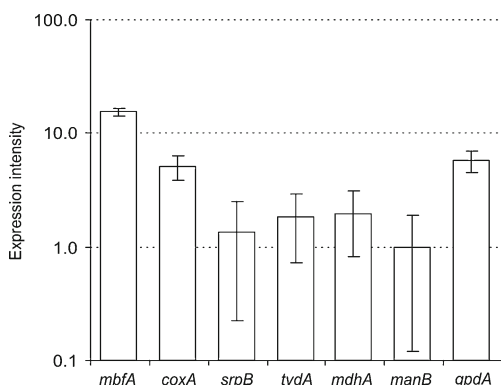
To estimate the transcription strength of the promoters, the transcription of the genes controlled by the six promoters was analyzed in batch cultures of A. niger strain ATCC 1015

(Fig. 1). According to the transcriptional analysis with DNA microarrays, the six promoters cover a range of one order of magnitude in transcription level with promoter P*manB* being the weakest and P*mbfA* the strongest promoter. Remarkably, the P*mbfA* promoter is significantly stronger than the characterized and widely used promoter of the *gpd*A gene. The *gpd*A transcription signal is in the range of the *cox4* transcription signal which is the second strongest promoter of the selected six.
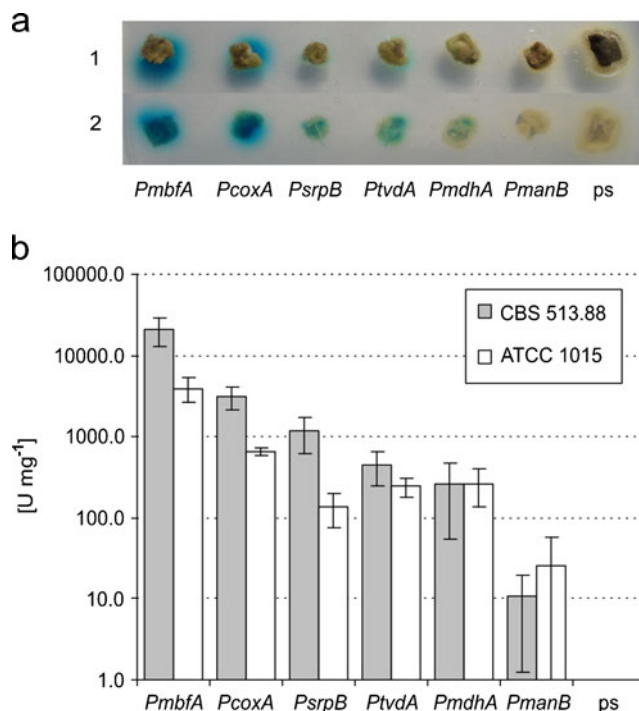
Promoter characterization using a *gusA* reporter system

Of the fragments located upstream of the coding sequences of the selected genes, 1.5 kb were PCR amplified and used as promoter regions in this study. To characterize the selected promoters for heterologous gene expression, cassettes with the GUS reporter gene under the control of the respective promoter were constructed and integrated into the genome of *A. niger* strains CBS 513.88 and ATCC 1015. Thus, the gene activity was directly observable on the agar plates for fungal growth (supplemented with X-Gluc; Fig. 2a). The blue color formation can be clearly seen for all promoter constructs except for P*manB*, which is indistinguishable from the parental strain.

A fluorimetric assay based on the conversion of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide allowed the precise quantification of the enzyme activity. The obtained values are shown in Fig. 2b. In all of the transformants carrying one of the six different promoter constructs, the GUS activity was detectable and significantly above the basal level of the parental strains, which were used as a negative control in all experiments. In case of ATCC 1015 transformants, the GUS activity correlates well with the transcription data and the order of the promoters is the same in both data sets being P*mbfA*> P*cox*A>P*mdhA*>P*tvdA*>P*srpB*>P*manB*. The six promoters

Fig. 2 Clones of *A. niger* expressing the *gusA* gene of *E. coli* under control of the six analyzed promoters. **a** Clones of *A. niger* CBS 513.88 growing on with agar solidified Vogel´s minimal medium supplemented with X-gluc. (1) front and (2) back side of the plate. *ps* parental strain CBS 513.88. **b** GUS activity (U (mg protein)$^{-1}$) in cell extracts of *A. niger* CBS 513.88 and ATCC 1015 clones containing the *gusA* gene of *E. coli* under the specified promoters. Strains were grown on MEA plates. Activity values are shown as arithmetic mean values with the standard deviation of at least two different clones, each measured in triplicate. The axis has a logarithmic scaling to the base 10

cover an activity range of two orders of magnitude. Interestingly, in case of CBS 513.88, the order of the promoter strength changes slightly, being: P*mbfA*>P*cox*A>P*srpB*>P*tvdA*> P*mdhA*>P*manB* (Fig. 2b). With three orders of magnitude, the range of activity between the strongest and the weakest promoter is even more pronounced in CBS 513.88. The GUS activity of a P*gpdA* construct showed a comparable activity as the *Pcox4* construct, thus confirming the initial transcription data (data not shown).

Application of the constitutive promoters in a metabolic pathway

A. niger is known to be a powerful producer of citric acid, however, it is naturally not able to produce itaconic acid, an industrially promising building block chemical. However, this organic acid can be produced in *A. niger* by expressing cis-aconitate decarboxylase (*cad1*) from *A. terreus*, as it was previously shown (Li et al. 2011). We took advantage of this system to demonstrate the usability of the characterized promoters in a metabolic pathway. For this purpose, the *cad1* gene of *A. terreus* was alternately put under control of one of

Fig. 1 Relative expression intensities measured with a custom DNA microarray (Agilent). The expression intensities were normalized to the expression value of *manB*, which was arbitrarily set to 1. The axis has a logarithmic scaling to the base 10. Strain *A. niger* ATCC 1015 was cultivated in a glucose mineral medium and RNA was extracted in the late exponential growth phase

the six promoters and the respective cassettes were integrated into the genome of the parental strain CBS 513.88. From each construct three randomly chosen colonies were tested for CAD activity (Fig. 3). CAD activity was found in all transformants tested and again the highest activity was found for strains carrying the P*mbfA* promoter construct whereas the lowest was detected using P*manB* promoter. The rank of the other 4 promoters is comparable to the results of the GUS activity assay. Only the construct with P*srpB* exhibits a lower CAD activity than expected, having this time slightly lower activity than the P*tvdA* construct.

The strains (at least three different clones of each promoter construct) were cultivated on Vogel's minimal medium to produce itaconic acid in baffled shake flasks. Itaconic acid was obtained for all cultivations except for the transformants carrying the *cad1* gene under the control of the very weak P*manB* promoter (Fig. 4). A maximal titer of 567 mg/L was obtained using the P*mbfA* construct which is comparable to previously published data (~700 mg/L) (Li et al. 2011).

In order to compare the CAD activity with the produced amount of itaconic acid, the specific productivity was calculated for each culture and plotted against the respective CAD activity value (Fig. 5). Figure 5 clearly shows the correlation of the CAD activity, which is dependent on the promoter, with the specific productivity of itaconic acid. The high deviation of the single measurements can be explained by the natural variance between the transformants as well as the inherently high variability of cultivation conditions in baffled shake flasks.

Discussion

For metabolic engineering purposes, it is crucial to have a powerful toolbox for the respective organism at hand. One
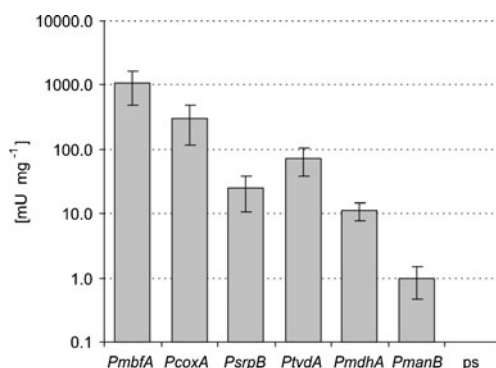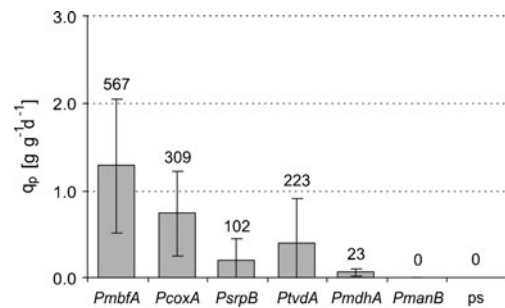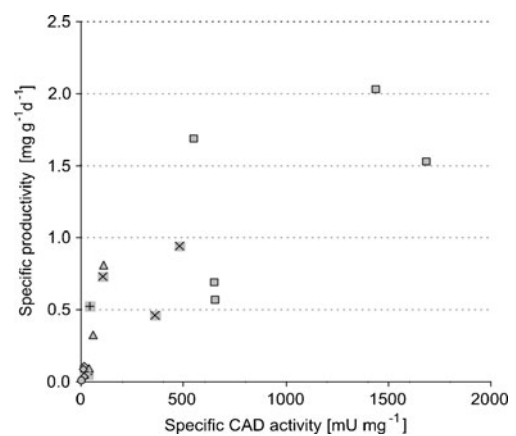

Fig. 4 Specific itaconic acid productivity q_P (in mg itaconate (g cell dry weight)$^{-1}$ (day)$^{-1}$)) of *A. niger* CBS 513.88 clones containing the *cad1* coding sequence of *A. terreus* under the specified promoter. The strains were cultivated in shake flasks on Vogel's minimal medium. Specific productivity values are shown as arithmetic mean values with the standard deviation of at least three different clones. The concentrations above the bars represent the maximal itaconic acid titer (in milligrams per liter) obtained with the respective construct. *ps* parental strain

important tool is well-characterized promoters in regard of expression strength and regulation. In this work we focused on the characterization of constitutive promoters with different expression strength. The NormFinder algorithm allowed us to explore all published transcriptomic data for the most stably expressed genes. Six promoters were chosen, namely P*mbfA*, P*coxA*, P*mdhA*, P*tvdA*, P*srpB*, and P*manB*, which gave rise to a wide range of expression levels in their natural genetic contexts as shown with transcription analysis with DNA microarrays. Subcloning these sequences in front of the *gusA* reporter gene confirmed their applicability also in a heterologous context. Consistently, the *mbfA* promoter led to the highest expression level, even significantly exceeding the activity of the *gpdA* promoter, which is currently the promoter of choice for high heterologous gene expression in *Aspergillus* spp. (Flaherty and

Fig. 5 Correlation between CAD activity (in mU (mg protein)$^{-1}$) and specific itaconic acid productivity (in g itaconate (g cell dry weight)$^{-1}$ (day)$^{-1}$)) of *A. niger* CBS 513.88 clones containing the *cad1* coding sequence of *A. terreus* under the control of the six analyzed promoters P*mbfA* (*squares*), P*coxA* (*x sign*), P*srpB* (*plus sign*), P*tvdA* (*triangles*), P*mdhA* (*diamonds*), and P*manB* (*circles*). Each data point represents the mean value of activity and specific productivity of measurements done in triplicates

Fig. 3 CAD activity (in mU (mg protein)$^{-1}$) in cell extracts of *A. niger* CBS 513.88 clones containing the *cad1* coding sequence of *A. terreus* under the specified promoters. Strains were grown on MEA plates. Activity values are shown as arithmetic mean values with the standard deviation of three different clones, each measured in triplicate. The axis has a logarithmic scaling to the base 10. *ps* parental strain

Payne 1997; Flipphi et al. 2001; Lombraña et al. 2004; Lubertozzi and Keasling 2008). Interestingly, the protein MbfA, encoded by *mbfA*, was not found in the intracellular proteome measured by Lu et al. (2010), whereas GpdA was found. On the one hand, this discrepancy can be explained by the measurement method used, which consisted of a 2D gel electrophoresis followed by a MALDI-TOF or Q-TOF detection. Not necessarily all proteins can be separated by this technique and detected and to our knowledge it was not shown that MbfA can be detected by this method. On the other hand, it can be speculated that the MbfA protein is very unstable and the actual concentration in the cell is low because of a massive turn-over cycle. However, further investigations are necessary to clarify this hypothesis.

The *manB* promoter showed consistently the lowest gene expression level, in all strains and construct combinations tested. The expression level of P*manB* was two or three orders of magnitude lower than that of P*mbfA*. The other four promoters gave rise to transcription and activity levels between these two. However, the order of the strength varied with different strains and different constructs.

Interestingly, the absolute expression values and obtained activities are generally and consistently higher for *A. niger* CBS 513.88 as compared with *A. niger* ATCC 1015. This finding is plausible considering the background, where these strains are used and derived from: *A. niger* CBS 513.88 is known to be a good protein producing host and is the ancestor of industrially important enzyme production strains (Pel et al. 2007). *A. niger* ATCC 1015 is known as the ancestor of acidogenic strains used for the production of organic acids (Andersen et al. 2011). Selection and laboratory evolution of these strains have therefore been quite distinct. While for CBS 513.88 strong transcription and translation is a key feature as the desired products are proteins, the constraints which have been important for ATCC 1015 have been different. Whatever makes a good acid producer might not be determined by a generally optimal protein expression.

The functionality of the new promoter set for metabolic engineering purposes was demonstrated in a case study using the target metabolite itaconic acid. Heterologous expression of the *cad1* gene of *A. terreus* in *A. niger* led to strains with an enzyme activity variation of three orders of magnitude. These strains accumulated itaconic acid in the supernatant to various degrees: While constructs with the strong P*mbfA* promoter produced up to 567 mg/L of itaconic acid under the tested conditions, no itaconic acid was detectable for P*manB* constructs. Intermediate concentrations for the other constructs proved the correlation of the promoter strength with the final titer. This correlation could be further shown by representing the specific productivity of different strains versus the enzymatic activity.

The highest achieved titer of 567 mg/L is in the same order of magnitude as the previously published value of ~700 mg/L

(Li et al. 2011), which underlines the usefulness of the new promoters. It has to be stressed in this context that the production conditions have not been optimized in our case, as we just wanted to verify the correlation of the promoter strength with the production of a useful metabolite. The slightly lower values compared with Li et al. (2011), which we reached can be explained by the different parental strain (AB 1.13) and the cultivation conditions (bioreactor system) used.

In line with our findings, that protein expression was generally more efficient in strain CBS 513.88 as compared with ATCC 1015, strain CBS 513.88 produced higher itaconic acid titers than strain ATCC 1015 (data not shown). Since we could prove that the productivity of itaconic acid strongly correlates with the *cad1* activity it does not come surprising that CBS 513.88 is the better producer under our tested conditions.

The presented constitutive promoters can be a valuable extension to a recently published inducible and tuneable expression system developed for *A. niger* (Meyer et al. 2011). With such a tuneable system (depending in this case on the inducer doxycycline) the optimal expression level for an enzyme could be determined. Subsequently, a constitutive promoter delivering the tailor-made expression intensity is applied for stable and robust gene expression. This has the advantage that the inducible system can be used for further rounds of experiments without interference of the already optimized enzyme activity and no additional, potentially harmful and/or expensive inducers are needed for industrial production processes.

Summarizing, in this paper, we characterized six novel constitutive promoters of *A. niger* and demonstrate their suitability as promoters for metabolic engineering purposes using the example of itaconic acid production.

Conflict of interest The authors declare that they have no conflict of interest.

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