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Author: Li, A.
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Chapter 2

A clone based transcriptomics approach for the identification of genes relevant for itaconic acid production in *Aspergillus*

An Li, Nicole van Luijk, Marloes ter Beek, Martien Caspers,
Peter Punt, Mariet v/d Werf

Abstract

Several *Aspergillus* species are well-known for the production of a variety of organic acids. In this study, a cloned based transcriptomics approach was used to identify genes crucial in the biosynthesis pathway for one of these acids, itaconic acid. From a number of different *A. terreus* controlled batch fermentations, those cultures with the largest difference in itaconic acid titer and productivity were selected for mRNA isolation. cDNAs derived from these mRNA samples were used for subsequent hybridization of glass slide arrays based on a collection of 5, 000 cDNA clones.
A selection of 13 cDNA clones resulting in the strongest (> 10 fold) differential hybridization signals were identified and subsequently the inserts of these clones were sequenced. Sequence analysis revealed the presence of in total 5 different gene inserts among the sequenced clones. From one of these sequences, encoding a gene of the MmgE-PrpD family, the full length coding region was shown to encode one of the crucial itaconic acid pathway enzymes *cis*-aconitate decarboxylase, by heterologous expression in *E. coli*. Expression of this gene in *A. niger*, which is a natural citric acid producer, resulted in itaconate production. Genome analysis suggests that in *A. terreus* the *cis*-aconitate decarboxylase gene is part of an itaconate acid related gene cluster including genes encoding two pathway specific transporters and a Zinc finger protein. Interestingly, this cluster is directly linked to the large lovastatin gene cluster.

Introduction

Itaconic acid (methylene succinic acid) (Fig 1) is an unsaturated C5 dicarboxylic acid, which can be used in industry as monomer or co-monomer in the manufacture of plastics, resins, synthetic fibers, paints, surfactant as well as elastomers. (Tate, 1981); (Milsom and Meers, 1985); (Okabe et al., 2009). Based on its industrial potential, it was selected by the Department of Energy in the US as one of the 12 building block chemicals, which are the most interesting compounds to be produced by industrial biotechnology (Aden A. et al., 2004).

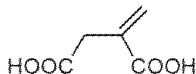


Figure 1. Structural formula of itaconic acid (molecular weight, 130.099 g/mole)

Itaconic acid production in *Aspergillus* was first found in *A. itaconicus* (Kinoshita, 1931). Later on, it was reported to be overproduced by *A. terreus* (Bonnarme et al., 1995). Currently, itaconic acid is produced commercially by overproducing classical mutants of *A. terreus* via submerged fungal fermentation (Okabe et al., 2009). The biosynthesis of itaconic acid in *A. terreus* occurs via the Embden-Meyerhof-Parnas (EMP) pathway and through the tricarboxylic acid (TCA) cycle. Citric acid is generally considered to be precursor of itaconic acid (Bentley and Thiessen, 1957). Due to the current price of itaconic acid (about US$ 2/ kg), its use is restricted (Okabe et al., 2009). In order to reduce the cost of itaconic acid, the production titer should increase from the current range of 70 - 90 g/l (Willke and Vorlop, 2001) towards the expected maximal theoretical yield in the range of 240 g/l. This yield is based on the yield of 360 g/l obtained for the commercial citric acid production process by *Aspergillus niger* (Tsao et al., 1999).

Therefore, based on its efficient citric acid production, *A. niger* was chosen as the production host strain for our research. *A. niger* has a long tradition of safe use (Schuster et al., 2002) in the production of enzymes and organic acids, and is widely used in biotechnology as host for the production of food ingredients, pharmaceuticals and industrial enzymes. In addition, *A. niger* grows on a wide range of substrates under various environmental conditions (Rumbold et al., 2009) (Pel et al., 2007).

The itaconic acid biosynthesis route in *A. terreus* has not yet been fully established. A complicating factor in this respect is that the pathway towards itaconic acid occurs in two compartments, in the cytosol and in the mitochondria. The proposed itaconic acid biosynthesis pathway starts with glycolysis in the cytosol. Glucose is metabolized to pyruvate mainly via the EMP pathway. Pyruvate is then either transported to the

mitochondria and dehydrogenated into acetyl-CoA, or carboxylated to oxaloacetate in the cytosol. Oxaloacetate is first converted to malate, and then converted back to oxaloacetate after being transported to mitochondria. In mitochondria, citrate synthase converts acetyl-CoA and oxaloacetate to citric acid. Further on in the tricarboxylic acid cycle (TCA), citrate is converted into *cis*-aconitate which is being transported back into cytosol and then decarboxylated to itaconate. A graphical presentation of the presumed pathway is given in Figure 2 (Jaklitsch et al., 1991). The efficient production of itaconic acid will depend on the sufficient fluxes of metabolites between two cell compartments, as was also addressed by Pedersen et al for amylase production in *A. oryzae* (Pedersen et al., 1999).

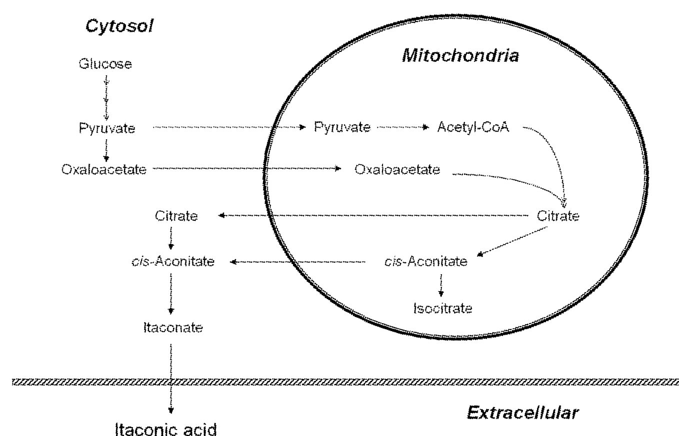


Figure 2. *Biosynthetic routes of itaconic acid in Aspergillus terreus.*

As can be deduced from this pathway, the yield of itaconic acid is depended on that the efficient production of citric acid, and its related biochemical pathway. Therefore it was expected that the itaconic acid pathway in *A. terreus* would rely on the genetic make up of the citric acid pathway with only a few additional genes, in particular genes encoding the last committed step from *cis*-aconitate into itaconic acid, *cis*-aconitate decarboxylase. Therefore in our research we decided to identify the itaconic acid specific pathway genes from *A. terreus* to allow their expression in *A. niger*.

In view of the relatively high titers, the itaconate biosynthesis is expected to be a dominant biological pathway in *A. terreus*. Most likely, the itaconic acid specific genes are expressed in elevated levels under itaconate-producing conditions, making a transcriptomics approach a

good option to identify these genes. In line with this, recently it has been demonstrated that transcriptomics is a valuable tool in identifying the relevant genes involved in primary metabolism (van der Werf et al., 2006). Therefore, in our study, we have used a transcriptomics based approach for identifying itaconic acid production related genes from *A. terreus*.

Materials and methods

Strains and plasmids

An isolate of *A. terreus* strain NRRL1960, was used for mRNA generation under various cultivation conditions, as well as chromosomal DNA extraction for gene amplification. *A. niger* strain AB 1.13 which derived from AB4.1 by UV irradiation (Mattern et al., 1992) was used as parental strain for *A. niger* transformation. The *Aspergillus* expression vector pAN52-4amdS derived by cloning the selection marker amdS into the vector pAN52-4 (EMBL accession #Z32699) was employed for *A. niger* gene expression. For construction of the *A. terreus* cDNA libraries, *E. coli* DH10b was used. *E. coli* strain DH5α used as the host in routine cloning experiments. *E. coli* strain BL21 (DE3) was used for expression studies in *E. coli*. The plasmid expression vector pET52 (Novagen®) was applied for expression of genes in *E. coli*.

Medium and growth conditions of *Aspergillus*

Fermentation of A. terreus for mRNA generation

In our experimental design, 12 batch fermentations (Table 1) of *A. terreus* were performed. The reference culture was based on a synthetic production medium as described previously (Riscaldati et al., 2000).

The production medium contained per liter 2. 36 g of NH_4SO_4, 0.11 g of KH_2PO_4, 2.08 g of $MgSO_4 \cdot 7H_2O$, 0.13 g of $CaCl_2 \cdot 2H_2O$, 0.074 g of NaCl, 0.2 mg of $CuSO_4 \cdot 5H_2O$, 5.5 mg of $Fe_2SO_4 \cdot 7H_2O$ and 1.3 mg of $ZnSO_4 \cdot 7H_2O$ and 0.7 mg of $MnCl_2 \cdot 4H_2O$. Glucose was supplied as carbon source, with 20 g/l for the pre-culture and 100 g/l for the main culture. All media were prepared in demineralized water. Before inoculation of the fermentors, *A. terreus* strain was precultured overnight in 500 ml baffled Erlenmeyer flasks with 100 ml of glucose production medium (10^7 spores/ml), 150 rpm, 37℃. Fermentations were performed in 5 liter Benchtop Fermentors (BioFlo 3000, New Brunswick Scientific Co., Inc.) at 37℃. The basic pH regime was initiated at 3.5 and subsequently fixed at 2.3, by 4M KOH (Base) and 1.5 M H_3PO_4 (Acid). In the basic DO (Dissolved oxygen) regime, DO was controlled at 75% on day1, at 50% on day 2, 3, 4 and at 25% on subsequent days (fermentation #1, 4, 10). The following variations were

applied to obtain variations in itaconic acid production. These variations were based on previously published data (Riscaldati et al., 2000) (Roehr et al. 1992) indicating effects on itaconic acid product of Mn concentration, C-source, pH and DO-control. In one fermentation culture (#9), 3.5 mg of $MnCl_2 \cdot 4H_2O$ (5 times higher) was used. One of the fermentation (#7) had a reduced glucose level of 30 g/l in the main culture. In two other fermentations fructose (#2) or maltose (#3) were used as the carbon source instead of glucose. In three fermentations #5,6, 11, a different pH control was applied. Fermentation #5 and #11 had a pH controled at 3.5 and 4.5 respectively, while fermentation #6 initiated at pH 3.5 after which pH was no longer controlled. In two fermentations # 8 and #12, an alternative DO regime was applied, namely 25% and 10% . Struktol (Schill & Seilacher) was applied as antifoam agent in all cultures throughout the fermentation.

Shake flask cultivation and fermentation of Aspergillus niger CAD transformants

For shake flask cultivation of *A. niger CAD* transformants, vogel's minimal medium (Vogel., 1956) was supplied with 2×100 ml per strain (10^6 spores/ml) in a 500 ml Erlenmeyer flask. *A. niger CAD* transformants were cultivated for 50-60 hours at 33℃, 150 rpm. The medium used for cultivating *Aspergillus niger CAD* transformants in the fermentor contained the same components as *Aspergillus terreus* with 0.7 mg of $MnCl_2 * 4H_2O$ and 100 g/l glucose as carbon source. Uridine (2. 5 g/l) was supplied to compensate the deficiency in the *A. niger* strain. Per fermentation, precultures were carried out in 2 × 100 ml of the production medium (10^6 spores/ml) in 500 ml baffled Erlenmeyer flasks for 64 hours at 33℃, 125 rpm. The fermentation conditions for *A. niger CAD* transformants were the same as the basic fermentation conditions for *A. terreus*, except that the DO was controlled at 25% throughout the fermentation.

Biomass dry weight measurement

Biomass of the batch cultivation was harvested by filtration of a fixed volume (10 ml) of the culture through a pre-weight filter and washed with demineralized water. Subsequently, the harvested biomass with the filter was dried in an oven at 110℃ over 24 hours and weighed. The biomass dry weight was established by subtracting the weight of the dry filter.

Transcriptomics approach

Microarray construction

The *A. terreus* clone-based array was constructed using cDNA generated

from the mRNA of *A. terreus* NRRL1960 via a polyT-primed RT-PCR reaction (Baseclear, Leiden, the Netherlands). The cDNA was size-fractionated to 1.0-1.5 kb fragments which were end-repaired and cloned into blunt-end pSMART-HC-kan vectors (Lucigen). A total of 5000 kanamycin-resistant colonies were picked into 96-well microtiter plates. Restriction analysis of 96 randomly selected clones and sequence analysis of 20 randomly selected clones were performed to check the size of insert, and confirm diversity and fungal origin respectively. Clone inserts were amplified by 96-well PCR (50 μl/well) using SMART-SR1/SL1 primers with 5'-C6-aminolinkers. PCR products were dissolved in 15 μl 3xSSC/well and spotted with quill pins (Telechem SMP3) on CSS100 silylated aldehyde glass slides (Telechem, USA) using a SDDC2 Eurogridder (ESI, Canada). During spotting, aminolinkers of PCR products will covalently link with aldehyde groups of the coated slides.

Hybridization
Total RNAs (5 μg/ 30 μl reaction), isolated from various *A. terreus* fermentation cultures with differential itaconate production, were labelled with amino-allyl-dUTP (aa-dUTP final concentration 0.75 μM, Sigma A0410) and reverse transcript (superscript II reverse transcriptase, Life Technologies) to aa-dUTP labelled cDNA. The purified aa-dUTP-labelled cDNA were coupled with Cy5-NHS-ester (Amersham). The reference DNA (genomic DNA) was labelled first with aa-dUTP then Cy3-NHS-ester (Amersham) using the Bioprime kit (Invitrogen). Array slides were prehybridized (with 5x SSC, 1% Bovine serum albumin, 0.1% sodium dodecyl sulphate) for 45 min at 42℃ and washed in MilliQ water. Spotted PCR products were denatured by boiling 5 sec in water and dried with N_2-gas. The Cy5- and Cy3-labelled sample were combined and denatured (2 min, 96℃) in 160 μl Easyhyb buffer (Roche, Cat # 11796895001), and then applied on a pair of prehybridised slides (slides A and B, 80μl/slide) at 50℃. Hybridization was performed overnight at 42℃. Slides were washed (1x in 1xSSC / 0.1% SDS 37℃, 1x in 0.5xSSC 37℃, 2x in 0.2xSSC 20℃) and dried with N_2-gas.

Image analysis and array data normalization
Slide images of Cy5- and Cy3-fluorescence were scanned by a ScanArray TM Express (Packard BioScience) and the intensity were quantified using Imagene 5.6 software (Biodiscovery).
Array normalization was done with several steps, signal (s) from all spots (Cy5 or Cy3) has been corrected for the background signal (b). S= s - b. All spots of S/b < 1.5 were temporally excluded for normalisation. Then, the average S_{Cy5} (avg S_{Cy5}) and S_{Cy3} (avg S_{Cy3}) were calculated for the remaining spots and the normalisation factor (n) was calculated as n =

avg S_{Cy5}/ avg S_{Cy3}. Rough spot ratios (r) were calculated (r = S_{Cy5}/ S_{Cy3}) and normalized (R = r / n).

Transcriptomics data analysis

The differential expression value was calculated by dividing the normalized S_{Cy5}/ S_{Cy3} ratio of a spot in the slide hybridized with RNA from the culture sample with the highest titer or productivity by the normalized S_{Cy5}/ S_{Cy3} ratio of that same spot in the slide hybridized with RNA from the culture sample with the lowest titer or productivity. In this analysis, titer is the amount of itaconate (g/l) produced from the selected *A. terreus* fermentation. Itaconate productivity at a certain time point was calculated as the slope of the regression line between that particular time point and the time points right before and after that time point. The fermentation profiles of the cultures used for the isolation of mRNA samples selected for the differential expression analysis are shown in Figure 3. The spots were subsequently ranked based on this ratio or, 1/ ratio when the ratio was < 1, i.e. in the case of down-regulated genes.

Expression of the CAD gene in *E. coli*

After identification of the putative *cis*-aconitate decarboxylase gene, a synthetic gene copy was cloned into *E. coli* expression vector pET52, resulting in vector pET-9971. Subsequently, *E. coli* strain BL21 (DE3) was transformed with plasmid pET-9971 and with vector pET52. The resulted transformants were cultivated in LB medium and on mineral salts medium (Hellmuth, 1994; Mattern et al., 1992), supplemented with 0. 5% glucose, 5 mM $MgCl_2$ and 100 μl/ml ampicillin. After cultivation at 37℃, 250 rpm to an OD600 of 0.5 - 0.8 and 30 min on ice, IPTG was added to a final concentration of 0.2 M. The cultures were subsequently cultivated overnight at 20℃, 250 rpm. Cells were harvested by centrifugation and the medium supernatant was analyzed by HPLC for the presence of itaconic acid.

Cloning and Expression in *A. niger*

The gene *cadA* which encodes *cis*-aconitate decarboxylase was cloned based on a PCR amplified gene copy derived from the used *A. terreus* strain. Two primers Cadfor53℃ 5′-TCCCGGATCCTTATACCAGTGGCGATTT-3′, Cadrev52℃ 5′-CCCCGGATCCTTATACCAGTGGCGATTTTACGG-3′ containing BspHI and BamHI restriction sites, respectively, were generated to amplifying the *cadA* sequence based on *A. terreus* genomic DNA. The complete coding region of the *cadA* gene was isolated from the PCR product as BspHI-BamHI fragments, and cloned into the *Aspergillus*

expression vector pAN52-4amdS. Subsequently, this vector was transformed into an *Aspergillus niger* strain AB1.13. The transformants resulting for this experiment were purified twice by single colony purification on AmdS+ selection plates (Kelly and Hynes, 1985). The chromosomal DNAs were isolated from 67 hours cultivated (minimal medium) *A. niger* AB 1.13 CAD transformants for Southern blot analysis (Okabe et al., 2009; Punt et al., 2008). Hybridisation was carried out using a probe derived from the *cadA* gene for probe labeling. The Alkphos direct labeling and detection system Amersham was used. As a loading control, a probe derived from the *A. niger gpdA* gene was used.

Extracellular organic acids and glucose quantification

The amount of extracellular organic acids and glucose was analyzed by a HPLC system equipped with two detectors, a Waters 2410 refractive index (RI) detector and a Waters 996 photodiode array (PDA) detector. An Aminex HPX-87H (Bio-Rad) sulfonic column was used for eluting compounds at 50℃ with 5 mM H_2SO_4 at a flow rate of 0. 5 ml/min. The glucose was detected by the RI detector while the organic acids were detected at 210 nm by the PDA detector. For quantification, glucose, citric acid, itaconic acid, oxalic acid and gluconic acid standards were run in parallel to the medium samples.

***Cis*-aconitate decarboxylase (CAD) activity assay**

Cell extracts were prepared by sonication (2 x 2 min sonication, setting 3, output 30-40% - Branson Sonifier 450) in 0.2 M sodium phosphate (pH 6. 5), 1 mM Na-EDTA, 1 mM DTT, 1 mM PMSF and 1.0 μg/ml pepstatin A. A cell free extract was obtained by centrifuging the crude extract for 15 min., 10.000 g, 4℃. These extracts were used for in vitro *cis*-aconitate decarboxylase activity assays (Jaklitsch et al., 1991).
The activity of CAD was determined by incubation of an aliquot of the crude cell extract at 37℃ for 30 min in 0. 2 M sodium phosphate containing 19 mM freshly prepared *cis*-aconitate at pH 6.2. The enzymatic reaction was terminated by addition of 25% HCl (1M solution). The denatured protein was removed by centrifugation and the supernatant was analyzed for itaconate formation by HPLC.

Results

Itaconic acid production under different growth conditions

Based on previous studies (Miall, 1978; Roehr et al., 1992), four key environmental conditions important for itaconic acid production in *A.*

terreus were identified. These are (i) carbon source, (ii) pH, (iii) trace element (i.e. Mn) concentration and (iv) dissolved oxygen (DO) tension. Fermentations with *A. terreus* were performed.

As shown in Table 1, a considerable variation in the amount of itaconic acid produced was observed, ranging from almost no itaconate (fermentation #11; pH 4.5) to about 50 g/l itaconate (#8 and #12; DO set point 25% and 10% respectively). Interestingly, the finding that low levels of DO results in a high itaconate titer is in contrast to what is reported in literature that itaconate production was induced under conditions of strong aeration (Roehr et al. 1992).

Table 1. Overview of the fermentations performed under different cultivation conditions. The reference fermentation was on 100 g/l glucose while DO was controlled at 75% in day 1, 50% in day 2-4, 25% from day 5 on. The pH was initiated at 3.5 and controlled at 2.3.

Fermen-tation	Environmental condition	Max. Itaconic acid (g/l)	Max. Biomass (gDWT/kg)
1	Glucose (100g/l) (reference)	16.1	12.7
2	Fructose as C- source	8.84	13.7
3	Maltose as C-source	13.9	12.1
4	Glucose (100g/l) (reference)	25.8	11.6
5	pH set 3.5	8.7	16.5
6	pH start 3.5 no set point	30.6	8.7
7	Low glucose (30g/l)	11.1	6.7
8	O_2 set point 25%	47.2	12.0
9	5×higher Mn	20.3	13.8
10	Glucose (100g/l) (reference)	26.9	17.9
11	pH set 4.5	0.1	20.4
12	O_2 set point 10%	52.9	10.6

In the course of every fermentation, 2 to 5 mycelium samples were harvested for mRNA isolation. Although it was not possible to isolate mRNA from fermentation #6 and #12, the remaining samples covered the complete range of itaconate production. For all samples at the time point where mRNA were isolated, the itaconate titer was determined and the productivity was calculated. Based on these results, mRNA samples from fermentations #4, #8 and #11 were selected for further analysis (Table 2).

Table 2. The itaconate titer and productivity of the selected samples used for mRNA extraction and the further transcriptomics analysis.

RNA sample Fermentation	Environmental conditions	EFT (hours)	Phenotype	Itaconic acid (g/l) titer	Productivity
4	pH set point 2.3	73	Low productivity	24. 00	-0. 15
8	O_2 set point 25%	54	High titer / High productivity	36. 00	0. 82
11	pH set 4. 5	52	Low titer	0. 04	0. 00

Our sample selection for transcriptomics study was based on the assumption, that the genes related to itaconate production will have a difference in expression in relation to itaconic acid titer or productivity. In contrast, the itaconate irrelevant genes are expected to express similarly in these conditions. As shown in table 2, the highest itaconate titer (36 g/l) obtained among all available samples with a corresponding mRNA sample, was derived from a fermentation controlled with reduced DO (25%) at 54 hours. This sample was also corresponding to a medium sample with the highest itaconic acid productivity. The mRNA sample corresponding to the lowest titer (0.04 g/l) was derived from a fermentation controlled at pH 4.5 at 52 hours. The lowest productivity (-0.15) was derived from the reference fermentation at 73 hours when itaconic acid levels (24 g/l) were no longer increasing. Figure 3 presents the relevant data from these three fermentation samples.

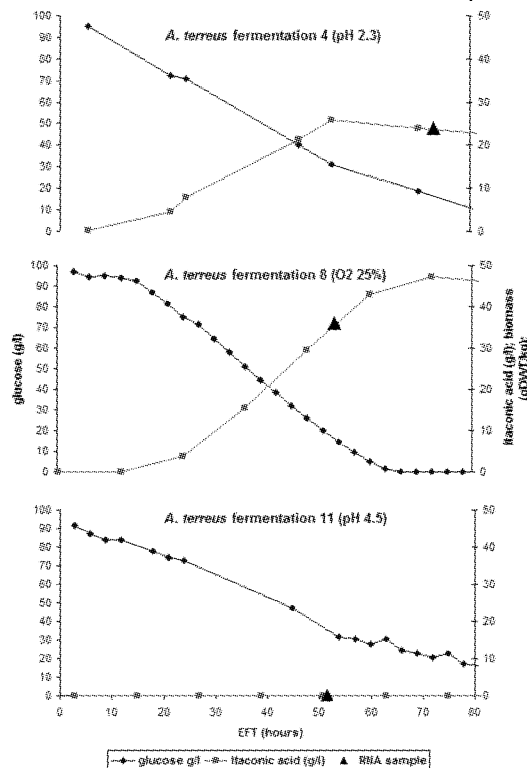


Figure. 3. *A. terreus* fermentation data of three different conditions used for the transcriptomics study. The RNA samples were isolated from mycelia harvest at 73 hrs, 54 hrs and 52 hrs respectively from fermentation 4, 8 and 11 indicated by a black tri-angle in each graph.

Identification of itaconate biosynthesis genes from transcriptomics study

Using the three isolated RNA samples, hybridization of cDNA based glass side arrays was performed. In order to identify the genes involved in itaconate biosynthesis from the normalized array data, the data were analyzed in a differential expression analysis approach. In this approach, the fold difference in expression between the condition of interest and a reference condition is used to identify the genes that are potentially of interest to the question of study. In our analysis, the fold difference in expression level was also used to rank the targets.

As the *A. terreus* array consist of two identical slides, the expression signal of the cDNA clones were averaged by taking the spots that gave a signal on both slides. Since we had no prior knowledge about regulation of the itaconic acid pathway genes, two different itaconic acid phenotypes were used for the differential expression analysis. One phenotype represents the itaconate production level (titer) and the other one represents the itaconate production speed (productivity). Based on these phenotypes, the expression ratios between the RNA corresponding to the fermentation sample with the lowest and the highest itaconate titer or lowest/highest productivity were calculated (Table 3).

For all clones, the expression ratios generated by differential analysis on itaconate titer or productivity were ranked. Those clones with a ratio above 10 in either of the two phenotypes were combined, and unique clones were identified. In total 13 spots obtained after the differential analysis were selected for sequencing (Table 3).

Tables 3. The itaconate biosynthesis related genes on the 13 highest overall ranking spots identified by differential expression analysis based on titer and productivity, respectively.

Clone ID	Array expression value (normalized)			Ratio	Rank	Ratio	Rank	Gene locus	Gene name/Remarks
	F4	F8	F11	titer		productivity			
AsTeR017E03	816.4	8889.5	263.1	33.79	1	10.89	6	ATEG_09970.1	predicted protein / Mitochondrial carrier protein
AsTeR037B09	1466.6	15066.8	484.4	31.10	2	10.27	7	ATEG_09971.1	predicted protein / MmgE/PrpD family
AsTeR008F12	319.6	3910.0	135.0	28.96	3	12.23	4	ATEG_09971.1	predicted protein / MmgE/PrpD family
AsTeR017E02	640.8	6550.4	321.8	20.36	4	10.22	8	ATEG_09970.1	predicted protein / Mitochondrial carrier protein
AsTeR027F02	640.2	6115.7	307.2	19.91	5	9.55	9	ATEG_09970.1	predicted protein / Mitochondrial carrier protein
AsTeR020B12	699.2	9987.6	616.4	16.20	6	14.28	1	ATEG_09970.1	predicted protein / Mitochondrial carrier protein
AsTeR031E12	873.5	11637.3	733.8	15.86	7	13.32	2	ATEG_09817.1	glyceraldehyde-3-phosphate dehydrogenase
AsTeR008H08	1142.4	6536.8	426.1	15.34	8	5.72	18	ATEG_09970.1	predicted protein / Mitochondrial carrier protein
AsTeR026D10	478.0	5544.2	381.1	14.55	9	11.60	5	ATEG_01954.1	predicted protein
AsTeR041A01	1707.1	2408.6	213.8	11.27	10	1.41	404	ATEG_00965.1	conserved hypothetical protein
AsTeR036C11	78545.9	64467.8	5991.9	10.76	11	0.82	517		rRNA
AsTeR025E11	1319.5	2085.9	197.3	10.57	12	1.58	330	ATEG_00965.1	conserved hypothetical protein
AsTeR005D11	437.0	5742.6	546.9	10.50	13	13.14	3	ATEG_09971.1	predicted protein / MmgE/PrpD family

As can be seen in Table 3, about 70% of the spots were present in the ‘top clones’ of both the itaconate titer and itaconate productivity differentials lists, pointing towards their relevance for itaconate production. Following sequence analysis of the 13 selected spots, the genes present on these inserts were identified by performing a homology

search using BLAST based on the *A. terreus* genome sequence as available from the BROAD institute (http://www.broad.mit.edu/annotation/fgi/).
As shown in Table 3, in the 13 clones we identified five different genes. Three of them were identified on multiple clones. Interestingly, two of these were corresponding to two neighboring genes (ATEG_09971 and ATEG_09970) in the *A. terreus* genome. The ATEG_09971 encodes a predicted protein which belongs to the MmgE/PrpD family, whose members are observed in a wide range of organisms including bacteria and fungi (Lohkamp et al., 2006). In *E. coli*, two enzymes, cis-aconitase and the prpD gene product 2-methylcitrate dehydratase are implemented in having citrate dehydratase activity (Brock et al., 2002). Based on this, we considered that in *A. terreus* the MmgE/PrpD protein (ATEG_09971) could be involved in conversion of *cis*-aconitate to itaconic acid. The neighboring gene (ATEG_09970) encodes a predicted mitochondrial carrier protein. Additionally, two genes were identified on single clones, which respectively encode glyceraldehyde-3-phosphate dehydrogenase and a predicted secreted protein. The remaining 3 clones were only identified based on the differential expression in relation to itaconic acid titer, not productivity. These corresponded to a conserved secreted protein (2 clones) and rRNA. Further analysis will be required to evaluate their putative role in itaconic acid production. To identify the final biosynthetic step from *cis*-aconitate to itaconic acid, we first focused on functional characterization of the MmgE/PrpD family gene.

Expression of gene ATEG_09971 in E. coli
In order to establish that the ATEG 9971 encoded protein is indeed responsible for *cis*-aconitate decarboxylase activity, an *E. coli*-codon-usage-optimized synthetic gene of the ATEG 9971 protein was generated and cloned into an *E. coli* expression vector, yielding vector pET-9971. After transformation, the *E. coli* transformants containing pET-9971 or a control vector (pET52 -without an insert) were cultivated in shake flasks on two different media in the presence and absence of IPTG induction.
Proteins were extracted from each strain and separated on a SDS-PAGE gel. IPTG-induced cells contained a distinct band with a molecular weight as expected for the CAD protein. HPLC analysis of the culture medium revealed that E.coli - pET 9971 produced itaconic acid in both rich medium and a purely synthetic medium after IPTG induction while this was not the case for *E. coli* containing the empty expression vector, or in cells not induced by IPTG (Table 4). Moreover, incubation of the cell extracts in the presence of *cis*-aconitate showed the conversion of *cis*-aconitate to itaconate, confirming the presence of *cis*-aconitate decarboxylase activity.

Table 4. Production of itaconate by cell extracts of an *E. coli* transformant containing the ATEG 9971 gene in expression vector pET52 cultivated on different media.

Strain	Medium	Itaconate in culture medium (mg/l)	Itaconate produced from *cis*-aconitate by cell free extracts (mg/h/mg protein)
E. coli - pET52 (empty vector)	Luria Broth + IPTG	0	0
E. coli - pET52 (empty vector)	Mineral salts medium + IPTG	0	0
E. coli - pET-9971	Luria Broth	0	0.4
E. coli - pET-9971	Luria Broth + IPTG	83	37
E. coli - pET-9971	Mineral salts medium	0	0
E. coli - pET-9971	Mineral salts medium + IPTG	56	18.6

Itaconate production in Aspergillus niger strain AB 1. 13 CAD

Based on the identification of the *A. terreus cis*-aconitate decarboxylase, in a further step to improve itaconic acid production in fungi, *A. niger* was chosen as the CAD expression host. This choice based on its efficient citric acid production and a long tradition of safe use. To obtain itaconic acid producing *A. niger* strains, the CAD gene provided with the constitutive *gpdA* expression signals (Punt et al., 1988) was transformed into *A. niger* strain AB 1. 13 (Mattern et al., 1992; Punt et al., 2008). Five colonies were randomly selected for shakeflask cultivation to analyze itaconic acid production and for the Southern blot analysis to verify the presence of the *cadA* gene in these strains.

Table 6. Shake flask cultivation characteristics of citric acid, itaconic acid, oxalic acid production by *A. niger* AB 1. 13 WT and five of its *cadA* transformants. Samples were taken 55-60 hrs with the dry weight per sample around 4 g/kg.

Strain	Glucose consumption* g/l	Citric acid g/l	Oxalic acid g/l	Itaconic acid g/l	Southern blot[a]
AB 1.13	10	0.85 (±0.14)	1.36 (±0.08)	0.00	-
CAD 5.1	10	2.07 (±0.25)	2.14 (±0.14)	0.09 (±0.004)	+ /-
CAD 7.2	10	0.55 (±0.16)	2.34 (±0.20)	0.07 (±0.004)	+ /-
CAD 10.1	10	0.54 (±0.17)	1.80 (±0.07)	0.13 (±0.009)	+ +
CAD 14.2	10	0.54 (±0.13)	2.09 (±0.21)	0.12 (±0.007)	+ +
CAD 16.1	10	0.56 (±0.17)	1.74 (±0.07)	0.13 (±0.010)	+ +

* Glucose was supplied with 10 g/l

[a] Relative score on copy number of *cadA* in Southern blot analysis

As demonstrated in Table 6, all 5 transformants produced itaconic acid

albeit at low levels. In figure 4, it is shown that all of the five AB 1.13 CAD transformants contain *cadA* gene copies whereas no *cadA* signal was observed with the wt *A. niger* strain AB 1. 13. Among all five transformants, two strains with the highest copies number and itaconic acid production in shake flasks (CAD10.1 and CAD16.1) were selected for cultivation in the *A. terreus* itaconate production medium. Parental strain *A. niger* AB 1.13 was cultivated under the same conditions as a negative control. For biomass determination, mycelium was harvested twice a day throughout the whole cultivation. Medium samples (supernatant) were collected by auto-sampling for itaconic acid determination. The medium samples were analyzed for the presence of organic acids by HPLC analysis (Fig. 5). The levels of itaconic acid, citric acid and oxalic acid in the three strains were compared. As shown, itaconate was detected in the cultivation medium of both transformants. In both strains, about 0.6 g/l itaconic acid was detected. There is no itaconate formation detected in parental strain AB 1. 13. All strains had quite similar glucose consumption and biomass formulation profiles (Table 7).

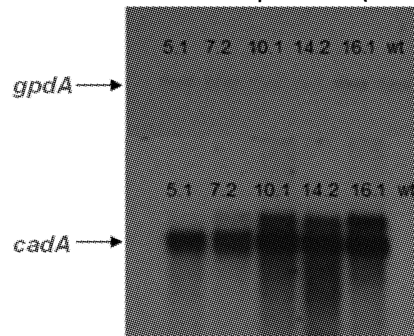


Figure 4. Southern blot analysis of the *A. niger* strain AB 1. 13 CAD transformants (5.1; 7.2; 10.1; 14.2; 16.1) and wt AB 1.13 as a negative control. The gpdA probe was used as a quality control for the analysis. The fragments labeled by gpdA probe (left) and CAD probe (right) are pointed with a black arrow.

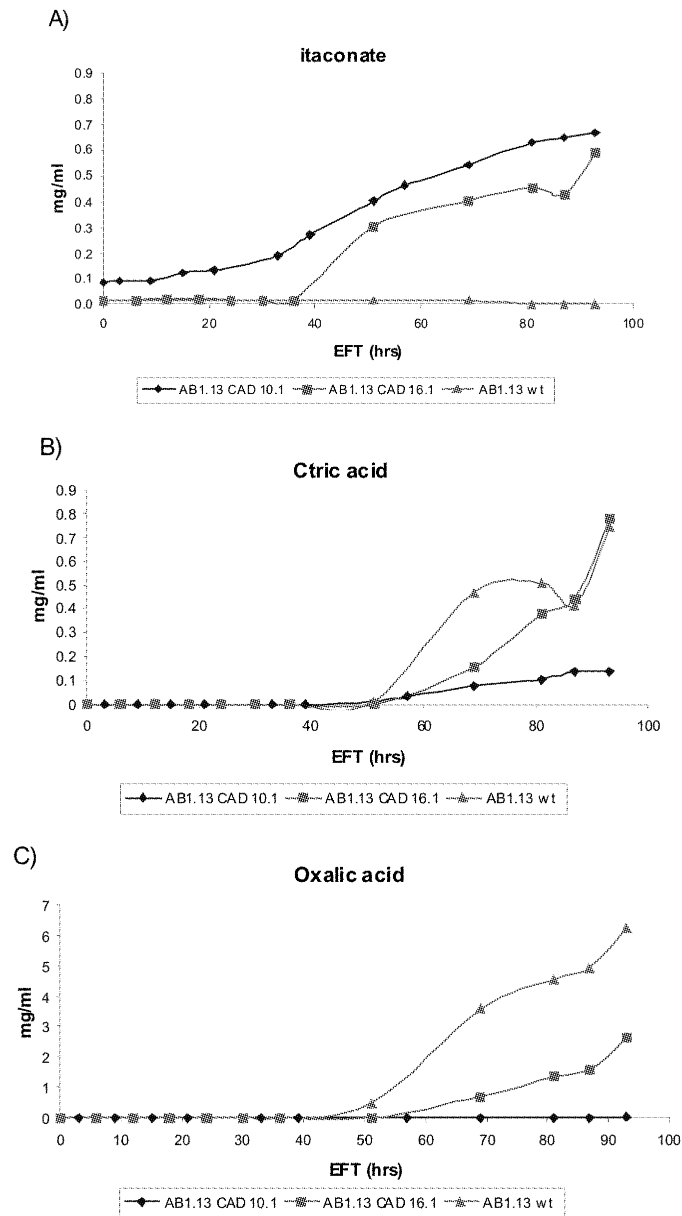


Figure 5. Itaconic acid, citric acid, oxalic acid production by *A. niger* AB 1.13 WT, AB 1.13 CAD 16.1 and AB 1.13 CAD 10.1 at pH 2.3. The fermentation duration for all three strains was 95 hours. (▲ , AB 1.13 WT; ■ , AB 1.13 CAD 16.1; ◆ , AB 1.13 CAD 10.1)

Table 7. Fermentation characteristics of glucose consumption, mycelium growth (Dry weight) as well as citric acid, itaconic acid and oxalic acid production by *A. niger* AB 1.13 WT and two of its *cadA* transformants at pH 2.3.

Strain	EFT hrs	Dry Weight g/kg	Glucose consumption* g/l	Citric acid g/l	Oxalic acid g/l	Itaconic acid g/l	% Ita.
AB1.13	90	18.70	60.20	0.70	6.00	0.00	0
CAD 10.1	90	18.00	63.80	0.15	0.00	0.70	82%
CAD 16.1	90	20.40	62.90	0.70	3.00	0.60	15%

* Glucose was supplied with 100 g/l

% Ita.- The percentage of itaconic acid among the three given organic acids.

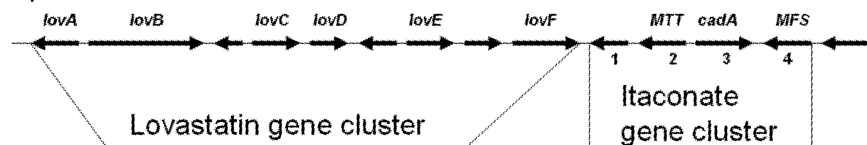
Interestingly, the three strains also showed differences in citric acid and oxalic acid production, which are the two major acids produced in wild type *A. niger*. Citric acid is the precursor of itaconic acid while oxalate can be considered as a waste product in the TCA cycle. Both transformant strains showed reduced levels of these acids where CAD 10.1 showed the most strongly reduced levels. In this strain about 85% of the produced acids consist of itaconic acid.

Discussion

As a result of a clone based transcriptomics approach, a key gene of the itaconic acid pathway in *A. terreus* was identified. Expression of this gene in *E. coli*, resulted in itaconate production, confirming its identity as *cis*-aconitate decarboxylase. During the work presented in this paper, the same gene was also identified using a completely different approach (Kanamasa et al., 2008). In our study, differed from the enzyme purification approach, the *cadA* gene was identified via a clone-based transcriptomics approach. To our knowledge this is the first time that a fungal biosynthesis pathway gene was identified in a transcriptomics approach using an experimental fermentation design aimed at largely different product levels. Based on our differential analysis, 3 out of 13 of the top ranking list were individual clones containing *cadA* gene sequences. By expressing this gene in *E. coli*, we further proved the presence of CAD activity originating from the cloned gene. In our view, the finding of multiple independent clones is a strong argument regarding the relevance of the identified gene, obviating further confirmation of the results by Northern or RT-qPCR analysis. Following the identification of the differently expressed *cis*-aconitate decarboxylase gene (*cadA*), experiments were initiated to express this gene in *A. niger* which is a natural citric acid producing strain, with the aim to convert it into a itaconic acid production host strain. In *A. terreus*, this acid is currently

produced at levels below theoretical yield, whereas industrial *A. niger* strains are capable of producing citric acid at theoretical yield. Therefore, we considered *A. niger* as a suitable candidate for itaconic acid, as this acid can be derived from citric acid via *cis*-aconitate (Fig. 2). Indeed, *cadA* gene expression resulted in itaconate production in the cultivation medium of *A. niger* transformant strains. As shown, not all secreted citric acid was converted into itaconic acid when comparing both strains analyzed. Apparently, the itaconic acid production medium used, which was developed for *A. terreus*, is not optimal for *A. niger*. In Table 7, it is shown that glucose was not fully consumed during batch fermentation. Further research in fermentation process optimization is currently ongoing. It should also be noted that the used *A. niger* strain is not a truly citric acid hyper producing strain. Therefore, further improvements in itaconic acid yields could be also obtained by further stain development, based on *A. niger* strains which already producing much higher levels of citric acid. As described, our clone based transcriptomics approach provided a practical platform for identifying genes related to primary metabolite production. Besides the gene encoding *cis*-aconitate decarboxylase, also 4 other itaconic acid production related genes were identified from this transcriptomics approach. One of these, which corresponded to 5 out of the 13 identified clones, gene ATEG_09970, was located directly adjacent to the *cadA* gene in *A. terreus* genome. This gene encodes a mitochondrial carrier protein. Based on genome mining, the encoded protein is suggested to function in transporting tri-carboxylic acids such as citric acid from mitochondria to cytosol and/or vice versa. This suggests that this genome region may consist of an itaconic acid gene cluster. Moreover, the other gene adjacent to *cadA* encodes a small solute major facilitator transporter (MFS). MFS transporters are a diverse family of transport proteins, transporting compounds ranging from sugars to organic acids, including dicarboxylic acids, of which in *A. niger* some 450 different members are present. The location of the MFS gene in the presumed itaconic acid gene cluster in *A. terreus* genome, suggest that it might be the itaconate exporter. Adjacent to the putative mitochondrial tricarboxylate transporter also a gene encoding potentially pathway specific binuclear Zinc finger protein is present (Fig. 6). Interestingly, this genomic region is directly linked to the lovastatin gene cluster (Sorensen et al., 2003), suggesting a link between both pathways, which are apparently specific for *A. terreus*. Also in the research of Lai et al., the production levels of itaconic acid and lovastatin are inversely correlated (Lai et al., 2007). To identify if the putative itaconate gene cluster is also present in other organisms, BLAST searches were performed. Although homologous

sequences for all four itaconic acid pathway genes could be identified in *A. niger*, only a low identity was found with the closest homologues in *A. niger* or any other organism for which a genome sequence was available, indicating that these homologous genes are not true orthologues. Therefore, the gene cluster seems indeed specific for *A. terreus*, and such a gene cluster is not present in *A. niger*, corresponding to the fact that among the fungal species for which extensive genome information is available only for *A. terreus* itaconic acid and lovastatin production is reported.



1: putative Regulator contains: -Zinc finger domain, -fungal specific transcription factor domain

2: putative Mitochondrial tricarboxylate transporter

3: *cis*-aconitate decarboxylase

4: putative Major Facilitator Superfamily transporter (transport small solutes)

Figure 6. *A. terreus* gene cluster, ATEG 09970.1 – ATEG 09972.1.

Based on the transcriptomics study and the genome mining, ATEG_09970 (putative mitochondrial tricarboxylate transporter) and ATEG _ 09972 (major facilitator superfamily transporter) are indicated as obvious target genes for further strain improvement towards itaconate production. Also further analysis of the role of the other genes identified in our transcriptomics approach may provide interesting aspects of the itaconic acid pathway. The role of the glyceraldehyde dehydrogenase ATEG_09817 may indicate an increased flux through glycolysis towards the TCA cycle. The possible relevance of increased glycolytic flux is also suggested by the results of Tevž (Tevž et al., 2010). These authors show that overexpression of a citrate inhibition resistant phospho-fructokinase (*pfkA*) gene result in incresed itaconic acid levels in *A. terreus*. Also, genes which were shown to be negatively involved in citric acid production in *A. niger* such as *goxC* (glucose oxidase) and *oahA* (oxaloacetate dehydrogenase) (Magnuson and Lasure, 2004; Ruijter et al., 1999), may also be potentially inhibitory for itaconic acid. Thus, deletion of these two genes in *cadA* expressing *A. niger* strains would be another possibility to increase itaconic acid productivity in *A. niger*.
The role of the two other proteins, ATEG_00965 and ATEG_01954, which encoding small secreted proteins remains fully to be established.
In our ongoing research, we will focus on both strain and process development towards itaconic acid production in *A. niger*.

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