Genetic engineering of *Aspergillus niger* for organic acid production

Dissertation

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Zusammenfassung

Die industrielle Biotechnologie eröffnet neue Perspektiven und Chancen für die Herstellung von Produkten aus nachwachsenden Rohstoffen. Wissensbasiertes Engineering von industriellen Mikroorganismen wird dabei immer wichtiger für die Stammverbesserung. Moderne genetische Methoden erlauben die Rekonstruktion und Modifikation von komplexen Stoffwechselwegen, um metabolische Muster, die Produktbildung oder die Robustheit der Zellen zu modifizieren. Die Entwicklung geeigneter genetischer Methoden für das Metabolic Engineering von industriellen Stämmen ist für weitere Fortschritte in der mikrobiellen Biotechnologie sehr wichtig.

Diese Arbeit konzentriert sich auf die Entwicklung einer genetischen Tool-Box für den filamentösen Pilz *Aspergillus niger*, der ein effizienter Produzent organischer Säuren ist. *A. niger* hat das Potential, einer der wichtigsten Wirtsorganismen für die Herstellung bio-basierter Chemikalien zu werden. Der erste Teil dieser Arbeit beschäftigt sich mit der Charakterisierung neuer konstitutiver Promotoren von *A. niger*. Diese Promotoren ermöglichen verschiedene Stärken der Genexpression und bilden eine hervorragende Grundlage für Metabolic Engineering. Der Einsatz dieser Promotoren im Stoffwechselweg zur Herstellung von Itaconsäure in *A. niger* führt zu neuen Stämmen, die diese Säure im Kulturmedium akkumulieren. Die Konzentration der Itaconsäure korreliert dabei mit der Stärke der Promotoren, was die Anwendbarkeit der Promotoren zeigt. Im zweiten Teil dieser Arbeit wurden die beiden Schlüsselenzyme für die Itaconsäurebiosynthese (cis-Aconitat Decarboxylase und Aconitase) gezielt in den Mitochondrien exprimiert, was zu einem maßgeblichen Anstieg der Produktion von Itaconsäure geführt hat. Diese Daten unterstreichen die Bedeutung der Enzym-Expression im richtigen Zellkompartiment, um Produktionsprozesse zu optimieren. Schlußendlich wurden im Laufe dieser Arbeit noch auf einem modifizierten AMA1 Fragment basierende Vektoren entwickelt, die eine transiente Transformation der Pilze ermöglichen.

Abstract

Industrial biotechnology is opening new perspectives and opportunities for the production of bio-based products from renewable resources. The rational engineering of industrial microorganisms becomes increasingly important for strain improvement. Advanced genetic techniques allow the reconstruction of complex networks of metabolic pathways and their modification to alter cell behavior, metabolic patterns and product formation. The development and implementation of appropriate genetic tools for genetic and metabolic engineering of industrial strains is essential for further progress in microbial biotechnology. This work focuses on development of a genetic tool-box for the filamentous fungus *Aspergillus niger* being an efficient organic acid producer. *A. niger* has a great potential to become one of the most powerful host organisms for the production of bio-based chemicals. The first part of this work aimed at the characterization of novel constitutive promoters of *A. niger* providing various expression levels of target genes as a powerful tool for metabolic engineering. Applying these promoters to the metabolic pathway for the production of itaconic acid in *A. niger* resulted in novel strains able to release itaconic acid into the culture medium. Itaconic acid concentrations correlated to the strength of the applied promoters, thus proving their functionality. In the second part of this work, the key enzymes of this metabolic pathway, cis-aconitate decarboxylase and aconitase were targeted into mitochondria which resulted in a substantial increase in the production of itaconic acid. This data highlights the importance of enzyme expression in the correct cellular compartment to establish an adequate flux of metabolites and optimize production processes. Finally, autonomously replicating transient vectors containing a modified AMA1 fragment as an origin of replication for fungi were developed.

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1. INTRODUCTION

1.1. Renewable resources

Nowadays, the majority of industrial products and processes are based on non-renewable resources and minerals. Petroleum and natural gas became a basic material for production of energy, transportation fuels, plastics, fertilizers, chemicals, and a wide variety of commodities including clothing, carpeting, computers, cosmetics, paint and other building materials (Polack et al. 2010). The demand on fossil sources is constantly increasing. The alarming dependency of our society on petroleum and natural gas contributes to the serious environmental, social and economic problems in our planet. Beside the fact that fossil fuels contribute to global warming through the emission of greenhouse gases, the most urgent problem is that world`s supply of fossil fuels is getting limited (Moore 2005; Piver 1991). Due to declining availability and constantly increasing price of fossil fuels, it is essential to establish solutions which reduce the rapid consumption of these non-renewable materials and find alternative, renewable resources.

A variety of alternative resources such as energy of sun, wind, water, biomass and geothermal energy can be established for energy production (Kamm et al. 2010). Currently, this renewable energy starts replacing conventional fuels for generation of power, heating and transportation fuels. On the other hand, the chemical industry and industrial biotechnology are opening new perspectives and opportunities for the production of bio-based products. Currently, industrial biotechnology focuses on three key directions for bio-based products. The first one concentrates on the substitution of oil and gas with biomass as raw material in the production of transportation fuels and chemicals (Scott et al. 2007).

Utilization of biomass instead of non-renewable resources can make a significant contribution to solving the problems of fossil sources shortages and their environmental impact. Raw materials as energy crops, municipal-, forest- or agro-waste are a cheap biomass sources that can be used for production of industrial products. Next strategy is based on the replacement of chemical synthesis by bioprocesses. The bioprocess refers to the use of live organisms to make a desired product within the pharmaceutical, food, cosmetics, fine chemicals and bulk chemicals sectors (Heinzle et al. 2007). In this process, the living cells or their components are applied on the production process, where carbohydrates are converted into amino acids, organic acids, alcohols, secondary metabolites such as antibiotics and other valuable chemicals. Finally, the third strategy aims at the development of novel bio-products with improved characteristics (Heinzle et al. 2007). All these strategies are tightly overlapping. As the biomass-derived carbohydrates are a great energy source for microbial fermentation, the bioprocesses can involve the use of new renewable starting materials for the production of industrial bio-products. Besides, microbes can be applied to produce novel substances (e.g. bio-plastics) that can replace petroleum-based materials.

At present, the main industrial bioprocesses include ethanol fermentation for fuels, as well as lactic acid or propanediol production for polymers (Kamm et al. 2010). Other examples of bio-based chemicals are non-food starch, cellulose fibers, fatty acids and citric acid (de Jong et al. 2012). However, in contrast to 330 million tons of chemicals and polymers produced from petrochemicals worldwide, the current global bio-based chemical and bio-polymer production (excluding biofuels) is estimated to be only 50 million tons (de Jong et al. 2012).

The key for the integrated production of food, feed, chemicals, materials, goods and fuels are biorefineries (Kamm et al. 2010). The biorefinery concept is analogous to today's petroleum refinery. But instead of producing multiple fuels and products from petroleum, they integrate advanced biomass conversion processes and equipment to produce these products from biomass. The biorefineries employ the biochemical, chemical and thermochemical processes. The most advanced biorefineries take advantage of the various components in biomass for the production of multiple products. Thus, almost all the types of biomass feedstocks can be converted to different classes of biofuels and biochemicals, while generating power and heat (Kamm et al. 2010; Cherubini 2010). However, the current biorefineries still focus on single technologies and feedstocks. The advanced biorefineries that could compete with existing industries are in the stage of development worldwide.

1.2. Microorganisms

Microorganisms have been used to produce various products for thousands of years. The early example of biotechnology is the production of wine and beer with microbial fermentation. Once scientists learned about the physiology and genetics of microbes, these organisms started to be frequently employed for biotechnological processes to generate industrially useful products (Glazer and Nikaido 2007). Due to their ability to utilize renewable raw materials, microorganisms are able to produce bio-based products such as organic chemicals, food and feed, detergents, textiles, polymers and bioenergy such as biofuels or biogas (OECD 2011). The major benefits of using microorganisms in biotechnology are easy handling and cultivation procedures thereby reaching an enormous number of cells. Furthermore, they are able to use a great variety of inorganic and organic

compounds as nutrients, reflecting an interesting metabolic diversity (Glazer and Nikaido 2007; Pelczar et al. 2009).

The bioprocess involving microbial cells or their components such as enzymes has many advantages over thermal and chemical processes. It requires low operating temperature and pressure, what is particularly important for production of compounds that cannot be made at high temperature. Living systems manage their chemistry more efficiently then chemical refineries due to high reactant specificity, product selectivity and minimal generation of hazardous wastes. Because enzymes have precise chemical selectivity, they may also use less purified raw materials. Furthermore, very often bioprocess requires milder conditions and fewer reaction steps than conventional methods, thus reducing energy and resource consumption (Vandamme 1994; Erickson et al. 2011). The possibility of having a scalable, controllable and economic process, in which renewable feedstocks are microbially converted to industrially useful products, opens promising perspectives for the future market.

However, the microbial bioprocesses still remain the challenge. Large scale industrial production is limited due to the difficulty to lower the production costs. Currently, bioprocessing is not as effective as chemical processing. Productivity, titer and yield very often do not reach their theoretical maximum. The other problems are unwanted by-products formation and high requirements for downstream separation and purification of final product. Furthermore, the manufacturing processes of natural resources are much more expensive and labor-intensive than those from crude oil-based materials. On the other hand, agriculture raw materials are becoming increasingly costly due to high demand for

biomass as raw material for production of bio-products, food, feed and energy as well as limited availability of the land (Chen 2012).

1.2.1. Filamentous fungi

Filamentous fungi have considerable potential as host organism for the production of variety of chemicals and extracellular enzymes. Some filamentous fungi have an enormous capacity to produce and secrete organic acids in high concentrations (Punt et al. 2002; Sharma 2009). *Aspergillus* is a one of the most efficient organic acid producers among all filamentous fungi. It is a promising host organism for production of bio-based chemicals from renewable resources. The main advantage is that *Aspergillus* is able to tolerate extreme cultivation conditions. It can grow at a wide range of temperatures (10–50°C), pH (2.0–11.0), osmolarity (from nearly pure water up to 34 % salt) water activity (0.6–1) and under oligotrophic or nutrient-rich conditions. Moreover, *Aspergillus* can degrade and utilize diverse biopolymers such as starch, (hemi-) cellulose, pectin, xylan and proteins (Meyer 2010). It also has the capability to continuously excrete large amounts of metabolites into the culture medium, while its morphology allows separation of cells by simple filtration, thus simplifying recovery of valuable products (Lubertozzi and Keasling 2009).

1.2.1.1. *Aspergillus niger*

A. niger is a widely used host organism for the industrial production of food processing enzymes and metabolites such as organic acids. The industrial production of citric acid based on *A. niger* fermentation was initiated in 1917, when James Currie discovered that this fungus is an efficient producer of citric acid. It was one of the first industrial fermentations in the world, which has been largely expanded and is applied to this day on an

enormous scale. Nowadays, citric acid is by far the major organic acid generated by fungal fermentation concerning production volumes. The industrial strains of *A. niger* are able to accumulate more than 200 g/L of citric acid during the production process. The commercial importance of this fungus is illustrated by a current global production of citric acid of 2 million tons (Arora et al. 1992; Berovic 2007; Glazer and Nikaidō 2007; Lubertozzi and Keasling 2009).

1.3. Metabolic engineering

The potential to use *A. niger* to produce other industrially important chemicals is huge. Next to further improvement of the production of naturally occurring compounds, the possibility to genetically modify organisms and exploit their properties to produce new substances have opened new opportunities for industrial biotechnology. Nowadays, genetic engineering allows adding genes that exist in totally unrelated organisms (Jarboe et al. 2010). On the other hand, the scientists have now the possibility to take a closer look at the mechanisms occurring in the cells. The constantly increasing numbers of publications, methods and data bases contribute to better understanding of physiology and morphology of organisms applied in biotechnology. Thereby the networks of complex metabolic pathways leading to the production of certain substances can be systematically analyzed. This knowledge allows employing recombinant DNA techniques to alter cell behavior, metabolic patterns and product formation (Lee and Papoutsakis 1999). Thus, the potential bottlenecks of the production pathways can be removed and resulting strains can gain new features that are essential to efficiently produce industrially important substances.

Metabolic engineering has a potential to enable the efficient microbial production of various chemicals from cheap and simple raw materials. Microorganisms can be engineered to produce significant amounts of desired products. On the other hand, natural utilization pathways can be modified or introduced from other organisms to enable using a variety of raw materials as carbon source. Optimized metabolic fluxes in the cell resulting in efficient production can be achieved by regulation or transferring of product-specific enzymes as well as entire metabolic pathways from genetically intractable organisms or even few different hosts to a favorite host strain (Keasling 2011).

In the last years, metabolic engineering has been successful for many applications. Thus far, the greatest part of the research has been performed on yeast (Abbott 2009; Sauer 2010) or bacterial host strains (Chen 2013; Zhang 2011). However, only a small part has been done on filamentous fungi. The achievements for *Aspergillus* species include e.g. reengineering of an azaphilone biosynthesis pathway in *A. nidulans* (Somoza et al. 2012), engineering of *A. niger* for itaconic acid production (Li et al. 2011) or D-galacturonic acid production (Mojzita et al. 2012), or engineering of the L-arabinose utilization pathway (Seiboth et. al. 2011).

1.3.1. Itaconic acid production with *A. niger*

In this work, we aim at the exploitation of the most efficient citric acid producer *A. niger* for itaconic acid production. In the last years, itaconic acid has become interesting future bio-based platform chemical. As an unsaturated dicarbonic organic acid, it can be incorporated into polymer chains and has found applications in the polymer industry. Additionally, it can be used as ingredient for the manufacture of synthetic fibers, coatings,

adhesives, thickeners and binders. It is non-toxic and readily biodegradable (Willke and Vorlop 2001; Okabe et al. 2009). Thus, itaconic acid is a promising substance that can replace petroleum-based chemicals widely used in detergents, plastics and other applications (Klement and Büchs 2012).

Each year, more than 80.000 tons of itaconic acid are produced worldwide (Okabe et al. 2009). However, the global demand for this acid is constantly increasing. As itaconic acid is still an expensive product with a price of $ 2 per kg, many potential applications are limited. The cheap production resulting in increased demand of itaconic acid could significantly broaden the horizon of the market. Currently, *A. terreus* is the most efficient host strain for the production of itaconic acid. Industrial strains can secrete around 80 g/L of itaconic acid to the media during submerged aerobic fermentation (Okabe et al. 2009). However, compared with the citric acid production with *A. niger*, the achieved titers are still low and the overall process is expensive. Further improvement of the itaconic acid production is still required to ensure cost-efficiency and enable to use it as a building block for bio-based chemicals in a large scale.

We attempt to exploit *A. niger* for itaconic acid production instead of further improving the fermentation process with *A. terreus*. We took advantage of *A. niger* being the most efficient citric acid producer. Citric acid is a precursor of itaconic acid. The significant concentration of itaconic acid could be achieved with this fungus, when the whole produced citric acid is utilized towards itaconic acid. In *A. terreus*, itaconic acid is generated via dehydration and decarboxylation of cis-aconitic acid. As *A. niger* lacks the enzyme converting cis-aconitic acid, the fungus is not able to produce itaconic acid. We reconstructed the metabolic pathway leading to itaconic acid production in this fungus (see Publications A and

B). We cloned the *cadA* gene of *A. terreus* encoding cis-aconitate decarboxylase and introduced it into *A. niger*. We succeeded in generating strains secreting itaconic acid into culture media.

1.4. Genetic tool-box

The rich history of using *A. niger* in biotechnology has resulted in the significant contribution to knowledge and understanding of this fungus. Nevertheless, for a long time, the genetic engineering of *A. niger* was very restricted. The *Aspergillus* research in relation to investigations of simpler yeasts and bacteria was remarkably delayed. It was caused by the complexity of the filamentous fungi such as multicellular morphology and thick chitinous cell wall, and led to the minor resources devoted to their study. Due to the lack of appropriate methods, the rational engineering of the industrial strains did not take place for a long time. The recent industrial processes using *A. niger* have been established by random mutagenesis and screening of highly selected industrial strains. Selection of best producing strains was not reproducible and mutations in the genome are not exactly known.

Currently, rational engineering becomes increasingly important for further strain improvement and generation of new strains gaining new features. Only the last decade of research has provided a large number of new data and fascinating discoveries regarding the use of *Aspergillus* as cell factory. It resulted in many new genetic tools and techniques including efficient genetic transformation systems, high-throughput gene targeting tools, expression systems for high level and controlled production as well as live-imaging techniques for cell biological studies (Meyer 2011).

1.4.1. Genetic manipulations

Currently, several laboratory techniques for *Aspergillus* transformation are available. The most common and effective method is based on the enzymatic digestion of the fungal cell wall followed by protoplast transformation. The protoplasts are incubated with transforming DNA in the presence of calcium ions and plated on selective osmotically-stabilized medium. Other methods include electroporation and a biolistic method (Ruiz-Díez 2002).

The transformation techniques require the use of selection markers. The broad range of currently available selection systems for *Aspergilli* includes antibiotic resistance markers (*hph*, *ble*, *oliC3*), nutritional markers (*amdS*, *ptrA*) and auxotrophic markers (*pyrG*, *pyrE*, *argB*, *adeA*, *adeB*, *niaD*, *trpC*, *sC*) (Meyer et al. 2011). The last group of markers requires first the generation of knock-out recipient strains, which can be further transformed with target DNA. Such a strain can be generated by homologous integration of foreign DNA into the exact locus. Homologous recombination enables precise disruption or replacement of genes of interest. However, in *Aspergillus* similar to most of the filamentous fungi, homologous recombination occurs at very low frequencies due to ectopic integration of the transforming DNA. Consequently, the generation of multiple knock-out strains is extremely time-consuming and labor-intensive. To overcome this obstacle, a system based on a deletion of the non-homologous end-joining (NHEJ) pathway, previously described for *Neurospora crassa* (Ninomiya et al. 2004) was applied. This deletion increases the homologous recombination frequencies up to 100 %. The inactivation of NHEJ pathway has been achieved by the knock-out of the *kusA* gene (Meyer et al. 2007) or the *mus-53* gene (Ishibashi et al. 2006).

Another possibility for genetic manipulations is using autonomously replicating plasmids. Extrachromosomal gene expression has many advantages over the integrative system. First of all, it allows avoiding random integration and unknown mutations in the genome. It provides high-frequency of transformation and enables recovery of insert-containing plasmid for further genetic analysis. Furthermore, transient plasmids that are stable only under selective conditions can serve as useful tool for genetic engineering. They can be used to express genes only for a limited period of time, and be removed from cells when taking away the selective pressure. Unfavorably, in contrast to yeasts and bacteria, filamentous fungi lack natural extrachromosomally replicating DNA elements. In 1991, Gems et al. isolated a 6.1 kb DNA fragment strongly enhancing the transformation efficiency and which enables to maintain autonomous replication. It has been demonstrated that this fungal origin of replication (AMA1) increases *A. nidulans* transformation efficiency up to 10^5 transformants per µg DNA (Gems 1991) without rearrangement of the plasmid, multimerization or integration into the host genome (Aleksenko and Clutterbuck 1997). In today's biotechnological applications plasmids containing the AMA1 region are frequently used for the transformation of filamentous fungi. However, the structure of the AMA1 fragment including two 3 kb long palindromic sequences and the short 0.3 kb central region is very problematic for genetic manipulations e.g. during the PCR amplification. In this study, we investigated shortened AMA1 fragments devoided one of the two palindromic sequences (see Manuscript C). We demonstrated that only one of the two palindromic sequences together with the central region of AMA1 is required to maintain autonomous replication in *A. niger*. The full-size of the remaining "arm" is necessary to provide high-transformation efficiency. Furthermore, we demonstrated that the plasmid containing "one-arm" AMA1 was removed from cells after at the latest in the 3rd generation when spores were cultivated

without antibiotic pressure. Such a system serves as powerful genetic tool for construction of transient vectors for temporary gene expression in *A. niger*.

1.4.2. Expression systems

Promoters play an essential role in controlling biosynthetic pathways. There are two main groups of promoters available for high-yield production of homologous and heterologous proteins in *Aspergillus* (Fleissner and Dersch 2010; Meyer et al. 2011). The first group includes inducible promoters which are dependent on the carbon or nitrogen source (e.g. the glucoamylase promoter P*glaA*, the alcohol dehydrogenase promoter P*alcA*, the a-amylase promoter P*amyA*), or are induced only in the presence of certain substances, like thiamine (thiamine promoter P*thiA*) (Shoji et al. 2005) or estrogen (human estrogen receptor *hERα*) (Pachlinger et al. 2005). These promoters are one of the easiest and most effective ways to regulate gene expression. However, in order to maintain strong gene expression, it is necessary to consistently induce activity of these promoters, what requires appropriate cultivation conditions or addition of expensive inducers. The second group includes promoters providing strong constitutive gene expression (e.g. the glyceraldehyde-3-phosphate dehydrogenase promoter P*gpdA*). Nevertheless, these promoters are focused on high expression level of the target gene.

Metabolic engineering demands for promoters which provide different strengths of expression. In fact, high gene expression not always results in high productivity, especially when the regulated gene controls an intermediate compound of a metabolic pathway. The optimization of complex networks of metabolic pathways requires fine-tuning and balancing of the involved activities. Actually, only one of the available expression systems for *Aspergillus* allows tight, tunable and carbon source-independent expression control. It is a

Tet-On/Tet-Off system which allows tunable gene control depending on doxycycline concentration (Meyer et al. 2011). It is tight under non-induced conditions is able to respond within minutes after inducer addition. However, industrial production demands for independence of addition of expensive inducers or other medium constraints. Construction of metabolic networks based on constitutively active genes allows for flexibility for microbial cell factory design. Constitutive promoters enable inexpensive, inducer-free gene expression, which is particularly important where cost is an issue. However, such 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 isolated and characterized six novel promoters of *A. niger* providing both, low and high constitutive expression of the target genes (see Publication A). Selected promoters demonstrated diverse expression levels of the *gusA* reporter gene in *A. niger* differing in a range of several orders of magnitude. To prove functionality of the promoters, we applied them in the metabolic pathway for itaconic acid production. Heterologous expression of the *cad1* gene of *A. terreus* in *A. niger* resulted in strains producing different amounts of itaconic acid in the culture medium, depending on the strength of the applied promoter. This set of promoters is a powerful tool for the precise tuning of gene expression that is required in metabolic engineering.

1.4.3. Cellular compartmentalization

The reconstruction of metabolic networks requires broad understanding of metabolic fluxes in the cell. The recent advances in research allow precise modeling of the complex metabolic pathways including competitive reactions and other constraints. However, many

metabolic engineering approaches do not take into account the cellular localization of the metabolites and enzymes involved in consecutive reactions. In fact, most of the proteins are engineered or overexpressed in the cytosol. However, many metabolic pathways are distributed over various cellular organelles, between which metabolites are continuously exchanged. Therefore, substrates and co-factor availability differ significantly within a cell. In this case, simple overexpression of enzymes in the cytosol could create substantial bottlenecks. The transport of intermediates across membrane can reduce productivity and enable these intermediates to be consumed by competing pathways. Consequently, transport processes play generally a crucial role, when analyzing and attempting to modify metabolic pathways. However, very little is known about transporters regulating metabolite exchange between cellular compartments.

The targeting of enzymes to the correct cellular compartment is another strategy to overcome the problem of cellular compartmentalization. Targeting proteins to the mitochondrium or other small cell organelles may result in significant improvement of the production of final metabolite. One of the benefits is greater local enzyme concentrations than in the cytosol. Thus, the availability of intermediates is also increased. They are concentrated in the small organelle and are not taken into competing pathways (Avalos et al. 2013). Recent studies emphasize the importance of compartmentalization of certain metabolic pathways. Wu et al. (2006) have shown that targeting of isoprenoid precursors into plastids elevates terpene production in plants. Recently, it has been shown that mitochondrial targeting of a whole isobutanol pathway improves the production of biofuels in *S. cerevisiae* (Avalos et al. 2013).

The itaconic acid biosynthetic pathway in *A. terreus* is also complicated by subcellular compartmentalization. The metabolic precursors for itaconic acid, citrate and cis-aconitate are produced in mitochondria. Further conversion with the cis-aconitate decarboxylase to itaconic acid takes place in the cytosol. The simple overexpression of the *cadA* gene of *A. terreus* in *A. niger* did not result in a high productivity. One reason for this might be the wrong compartmentalization of the enzymatic activities. Therefore, we set out to target the heterologous itaconic acid production pathway into the mitochondrium of *A. niger*. We investigated the effect of simultaneous overexpression of cis-aconitate decarboxylase and aconitase in the same compartment of the cell, cytosol and mitochondrium. We demonstrated that moving the enzymes into mitochondria resulted in a substantial increase in the production of itaconic acid (see Publication B).

This data show that targeting of enzymes to the correct compartment is an important means for metabolic engineering. Subcellular metabolic engineering has the potential to provide multiple mechanisms to improve the performance of engineered pathways.

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3. AIM OF THE STUDY

This work was part of the *FHplus* project "METORGANIC" focusing on metabolic analyses and metabolic engineering of industrially relevant fungi. Rational genetic engineering of *A. niger* is a promising way to use renewable resources for production of organic acids, and to significantly improve industrial production processes. For this purpose, an implementation of appropriate genetic tools for metabolic engineering is essential.

In this study, we isolated and characterized a set of promoters providing constitutive expression of the target genes on varying expression levels. We applied them on the metabolic pathway leading to itaconic acid production in *A. niger*. These promoters are a powerful tool for metabolic engineering purposes. They can be used for the fine tuning of metabolic pathways and testing the influence of gene expression levels at the industrial production processes.

Furthermore, we engineered the metabolic pathway for itaconic acid production in *A. niger*. We showed that targeting of the key reaction enzymes to the mitochondrium significantly improves the production of itaconic acid. These data highlight that expression of enzymes in a correct cellular compartment is crucial to establish a proper flux of metabolites and optimize production process.

Finally, we modified an autonomously replicating element for filamentous fungi (AMA1) and investigated its stability. We proved that shortened AMA1 fragment maintains its replicating properties without reducing transformation efficiency. The plasmid was removed from the cells as soon as taking away the selective pressure. Modified fungal origin of

replication enables quick and simple construction of self-replicating expression cassettes. Such a system can be used as a powerful transient expression tool for genetic engineering purposes in *A. niger*.

4. OUTLOOK

The bio-products represent a promising branch of industrial biotechnology. Advanced products made from renewable resources instead of petrochemicals are hitting the market today. The processes involving microorganisms in the production of bio-based chemicals have the capacity to rival chemical and thermal processes in the near future. The presented genetic engineering strategy for *A. niger* is a promising way to significantly improve the production of natural metabolites as well as heterologous substances with this fungus. As *A. niger* is an excellent citric acid producer, it has a potential to efficiently produce other chemicals in controllable, economic processes. The developed genetic tools including constitutive promoters of different strength, mitochondrial signal sequences and transient extrachromosomal vectors can contribute to the improved performance of metabolic pathways and can be beneficial for industrial microbial systems.

This work presents an example of metabolic engineering where *A. niger* was engineered to heterologously produce itaconic acid. *A. niger* has a great potential to become a powerful producer of this industrially important chemical. Itaconic acid is a promising substance with regard to replacement of petroleum-based chemicals, thereby reducing negative impact on environment and problems of fossil resources shortages. The economic itaconic acid production process can increase the availability of this acid and thereby create additional markets and increase the number of potential applications. The introduction of the itaconic acid synthesis pathway of *A. terreus* to *A. niger* is a great starting point for further investigations. Although the targeting of this metabolic pathway to mitochondrium significantly improved the productivity, it did not fully eliminate the bottleneck in this

process. The engineering of regulatory and transport mechanisms of this pathway is regarded as the second phase of metabolic engineering. Further studies should focus on the transport of metabolites between cytosol and mitochondrium. The overexpression of certain transporters located in the same gene cluster could result in a proper flux of metabolites and significant production improvement. Further investigations of itaconic acid production pathway in *A. niger* are highly recommended to establish effective itaconic acid production systems that could compete with existing processes in industry.

5. PUBLICATIONS AND SCIENTIFIC MANUSCRIPTS

5.1. Publications of this study

A. Blumhoff M, Steiger MG, Marx H, Mattanovich D, Sauer M (2013) Six novel constitutive promoters for metabolic engineering of *Aspergillus niger*. Appl Microbiol Biotechnol. Jan;97(1):259-67.

B. Blumhoff ML, Steiger MG, Mattanovich D, Sauer M (2013) Targeting enzymes to the right compartment: metabolic engineering for itaconic acid production by *Aspergillus niger*. Metab Eng. May 29;19:26-32.

5.2. Scientific manuscripts

C. Blumhoff ML, Kiesswetter U, Steiger MG, Schindler K, Sanystra C, Mattanovich D and Sauer M (2013) Autonomously replicating plasmids as a transient expression tool in *Aspergillus niger*. Manuscript in preparation.

D. Blumhoff M, Steiger MG, Kiesswetter U, Marx H, Mattanovich D, Sauer M (2011) Tool box for the transformation of industrial filamentous fungi. 5. Forschungsforum der Österreichischen Fachhochschulen – conference proceedings. FH Campus Wien, Wien May 27.-28.

5.3. Other publications

E. Steiger MG, Blumhoff ML, Mattanovich D, Sauer M (2013) Biochemistry of microbial itaconic acid production. Front Microbiol. 4:23.

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

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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.

Keywords Promoter study · *Aspergillus niger* · Metabolic engineering · Genetic engineering · Heterologous protein expression · Itaconic acid

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

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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 $2\times H_2O$, 5 g/L KH_2PO_4, 2 g/L NH_4NO_3, 0.2 g/L $MgSO_4$ $7\times H_2O$, 0.1 g/L $CaCl_2$ $2\times H_2O$, 0.5 mg/L citric acid $1\times H_2O$, 0.5 mg/L $ZnSO_4$ $7\times H_2O$, 0.1 mg/L $Fe(NH_4)_2$ $(SO_4)_2$ $6\times H_2O$, 0.025 mg/L $CuSO_4$ $5\times H_2O$, 0.005 mg/L $MnSO_4$ $1\times H_2O$, 0.005 mg/L H_3BO_3, 0.005 mg/L Na_2MoO_4 $2\times H_2O$, 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 protoplasts were resuspended in STC and directly used for transformation. 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 incubation 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 transformants 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 genomic 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 phosphate 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 fluorescence was measured at excitation/emission of 355/460 nm with the time interval of 80 s. The linear range of fluorescence 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 measured according to Bentley and Thiessen (1957). The reaction 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_2SO_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_2SO_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 possible different conditions. In order to find constitutive promoters 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 software was originally developed to find stably expressed reference genes for quantitative PCR analysis. The underlying algorithm calculates which gene shows the lowest variance 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*coxA*>P*mdhA*>P*tvdA*>P*srpB*>P*manB*. The six promoters

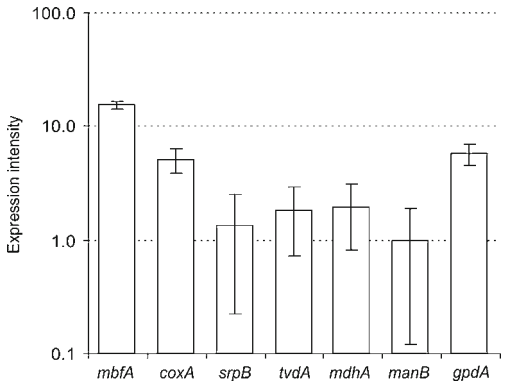

Fig. 1 Relative expression intensities measured with a custom DNA microarray (Agilent). The expression intensities were normalized to the expression value of *manB*, which was arbitrarily set to 1. The axis has a logarithmic scaling to the base 10. Strain *A. niger* ATCC 1015 was cultivated in a glucose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

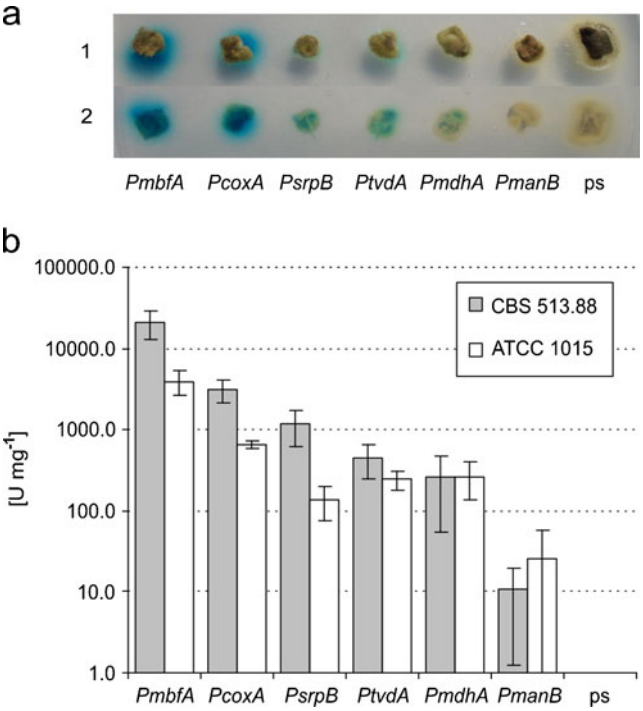

Fig. 2 Clones of *A. niger* expressing the *gusA* gene of *E. coli* under control of the six analyzed promoters. **a** Clones of *A. niger* CBS 513.88 growing on with agar solidified Vogel´s minimal medium supplemented with X-gluc. (1) front and (2) back side of the plate. *ps* parental strain CBS 513.88. **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

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 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

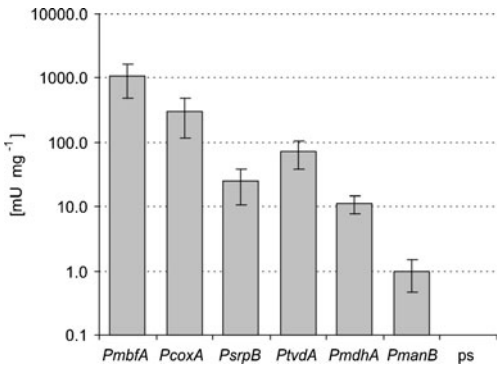

Fig. 3 CAD activity (in mU (mg protein)$^{-1}$) in cell extracts of *A. niger* CBS 513.88 clones containing the *cad1* coding sequence of *A. terreus* under the specified promoters. Strains were grown on MEA plates. Activity values are shown as arithmetic mean values with the standard deviation of three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

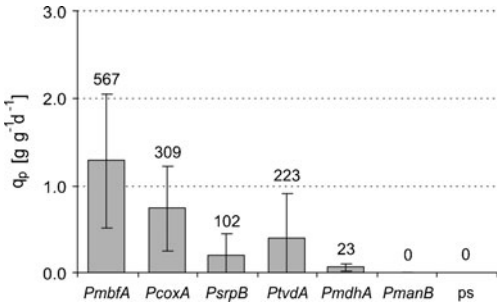

Fig. 4 Specific itaconic acid productivity q_P (in mg itaconate (g cell dry weight)$^{-1}$ (day)$^{-1}$)) of *A. niger* CBS 513.88 clones containing the *cad1* coding sequence of *A. terreus* under the specified promoter. The strains were cultivated in shake flasks on Vogel's minimal medium. Specific 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

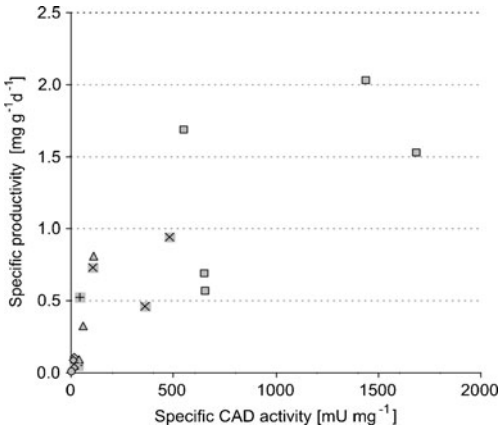

Fig. 5 Correlation between CAD activity (in mU (mg protein)$^{-1}$) and specific itaconic acid productivity (in g itaconate (g cell dry weight)$^{-1}$ (day)$^{-1}$)) of *A. niger* CBS 513.88 clones containing the *cad1* coding sequence of *A. terreus* under the control of the six analyzed promoters P*mbfA* (*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

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.

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

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Targeting enzymes to the right compartment: Metabolic engineering for itaconic acid production by *Aspergillus niger*

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ABSTRACT

Itaconic acid is an unsaturated dicarboxylic acid which has a high potential as a biochemical building block. It can be microbially produced from some *Aspergillus* species, such as *Aspergillus itaconicus* and *Aspergillus terreus*. However, the achieved titers are significantly lower as compared to the citric acid production by *A. niger*. Heterologous expression of *cis*-aconitate decarboxylase in *A. niger* leads to the accumulation of small amounts of itaconic acid. Additional expression of aconitase, the second enzyme metabolically linking citric acid and itaconic acid improves productivity. However, proper organelle targeting of the enzymes appears to be an important point to consider. Here we compare the mitochondrial expression with the cytosolic expression of *cis*-aconitate decarboxylase or aconitase in *A. niger*. Heterologous expression of both enzymes in the mitochondria doubles the productivity compared to strains which express the enzymes in the cytosol. It is essential to target enzymes to the correct compartment in order to establish a proper flux through a compartmentalized pathway.

1. Introduction

Itaconic acid (2-methylidenebutanedioic acid) is an unsaturated di-carboxylic acid with a broad application spectrum as a building block chemical (Willke and Vorlop, 2001; Okabe et al., 2009). Particularly for the production of polymers, itaconic acid is of interest, because it has the potential to function as a substitute for acrylic and methacrylic acid (Okabe et al., 2009). However, these applications require a very low price of the starting material to be economically competitive, which is currently not the case. Presently, the market price is about $2/kg and the worldwide production capacity of itaconic acid is expected to be about 50 kt/year, facing a demand of about 30 kt at the current price (Steiger et al., 2013).

A variety of itaconic acid producing microorganisms such as *Ustilago zeae* (Haskins et al., 1955), *U. maydis*, *Candida* sp. (Tabuchi et al., 1981) and *Rhodotorula* sp. (Kawamura et al., 1981) have been identified. However, currently, itaconic acid is exclusively produced by fermentation with *Aspergillus terreus* (Willke and Vorlop, 2001), which is able to secrete substantial amounts of the acid into the media (> 80 g/L). However, compared with the citric acid production process by *Aspergillus niger*, which accumulates more than 200 g/L, the achieved itaconic acid titers are still low. As a consequence, the possible range for improving itaconic acid titers appears to be substantial.

Only two enzymatic steps separate itaconic acid from citrate metabolically: aconitase (ACO) catalyzes the dehydration of citrate to *cis*-aconitate, which is decarboxylated by *cis*-aconitate decarboxylase (CAD) to itaconate. As aconitase is a component of the TCA cycle, it is a ubiquitous enzyme which is also present in *A. niger*. Thus making use of its native aconitase activity, *A. niger* can also produce itaconic acid solely overexpressing the *cadA* gene from *A. terreus* (Li et al., 2011, 2012; Blumhoff et al., 2013). However, the efficiency for itaconic acid production is very low compared to natural products like citric, oxalic or gluconic acid.

One reason for this might be the wrong compartmentalization of the enzymatic activities. Citrate is produced in mitochondria. The anti-port of malate and citrate is believed to be one of the motors for the efficiency of the citric acid production process (De Jongh and Nielsen, 2008; Röhr and Kubicek, 1981). In *A. terreus* and *A. niger* aconitase exists primarily in mitochondria (Jaklitsch et al., 1991a, 1991b). CadA is exclusively located in the cytosol (Jaklitsch et al., 1991b). The transport reactions necessary for itaconate production have not been characterized in detail (Steiger et al., 2013). However, the *cadA* gene of *A. terreus* is located in a gene cluster together with two transporters (one a putative mitochondrial carrier, the other a putative plasma

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membrane carrier). Overexpression of either the mitochondrial carrier or the plasma membrane carrier led to an increased itaconic acid production in *A. niger* (Li et al., in press; Jore et al., 2011). However, the overexpression of the plasma membrane carrier in a strain already expressing the mitochondrial carrier did not yield a further increase in itaconic acid titers (Li et al., 2013).

Here, we suggest another strategy: namely the co-overexpression of aconitase and *cis*-aconitate decarboxylase in the same compartment of the cell. Citrate is present in both compartments as it is produced in the mitochondria and transported via the cytosol to the medium. While targeting aconitase to the cytosol is relatively straight forward, the functional expression of a heterologous enzyme in mitochondria of *A. niger* has not been shown before.

Kirimura et al. (2006) constructed an *A. niger* transformant strain expressing a fusion of the mitochondrial alternative oxidase (*aox*) and enhanced green fluorescent protein (eGFP) to visually analyze the expression of *aoxA*. They could confirm the localization of the fusion protein in the mitochondria by co-staining with MitoTracker Red. eGFP fused with the mitochondrial-targeting signal of citrate synthase (AoCit1) was used to visualize the mitochondrial structures of *A. oryzae* (Mabashi et al., 2006). Similarly, the citrate synthase N-terminal region was used to target various fluorescent proteins to the mitochondria of *A. nidulans* (Suelmann and Fischer, 2000; Toews et al., 2004).

Retargeting of mitochondrial enzymes to the cytosol for metabolic engineering purposes was shown for example for malic enzyme in *Saccharomyces cerevisiae* (Moreira dos Santos et al., 2004) or different TCA related enzymes in *A. niger* (De Jongh and Nielsen, 2008) and *S. cerevisiae* (Xu et al., 2012). Targeting metabolic pathways to organelles was applied in plants, where parts of the isoprenoid pathway have been successfully targeted to plastids of the tobacco plant (Wu et al., 2006). Recently, it has been shown that mitochondrial targeting of a whole pathway is beneficial for the production of branched-chain alcohols in *S. cerevisiae* (Avalos et al., 2013). Here, we set out to target the heterologous itaconic acid production pathway to the mitochondria as well as to the cytosol of *A. niger*.

2. Materials and methods

2.1. Strains and media

Escherichia coli TOP10 was used as host for recombinant DNA transformation. *A. niger* ATCC 1015 was used as parental strain for *A. niger* transformations. *E. coli* transformants were cultured on LB and *A. niger* on ME/MEA or Vogels' minimal medium as previously described (Blumhoff et al., 2013) except that Vogel's medium was supplemented with 20% glucose and without the addition of $MnSO_4$. ME/MEA were supplemented with 150 μg/mL hygromycin or 100 μg/mL zeocin.

2.2. Construction of expression cassettes

Plasmids were constructed using an enzymatic assembly method (Gibson et al., 2009). Target DNA fragments were obtained as previously described (Blumhoff et al., 2013). The primers used for PCR amplification are specified in Table S1 in the Supplementary Material. Expression cassettes are composed of a promoter, the coding sequence of the target gene and a terminator. The coding region optionally includes a mitochondrial targeting sequence (MTS). The promoters, P*mbfA* of *A. niger* (Blumhoff et al., 2013) or P*icdA* of *A. niger* (941 bp upstream region of *icdA* gene) (Kirimura et al., 2002) were amplified from genomic DNA of strain ATCC 1015. MTS regions, initial 84 amino acids of *icdA* of *A. niger* (Kirimura et al., 2002) or initial 24 amino acids of *acoA* of *A. niger* (JGI 52568) predicted with MitoProt (Claros and Vincens, 1996), were amplified from genomic DNA of strain ATCC 1015 (see Fig. S1 in the Supplementary Material). The *cadA* (*cad1*) coding sequence of *A. terreus* was amplified from pCAD_*mbfA* (Blumhoff et al., 2013). The *eGFP* was amplified from pET30a_GFP-mut3.1 (Stadlmayr et al., 2010).The *DsRed* was amplified from pRHN1 (Janus et al., 2007), kindly provided by Ulrich Kück, Ruhr-Universität Bochum. The *acoA* gene of *A. niger* (JGI 52568) was amplified from genomic DNA of strain ATCC 1015. The *acnA* gene of *E. coli* (Gene Bank YP_001458101.1) was amplified from genomic DNA of TOP10 strain. The *ACO1* gene of *Saccharomyces cerevisiae* (Gene Bank M33131.1) lacking the initial 16 amino acids in order to provide cytosolic protein localization (Regev-Rudzki et al., 2005) was amplified from genomic DNA of CEN.PK 113-5D strain. The *TtrpC* terminator was amplified from pAN52-1N (Gene Bank Z32697). The plasmid backbone contained the hygromycin resistance cassette composed of the *hph* gene under the P*pki* promoter and the T*cbh2* terminator amplified from pCAD_*mbfA* and origin of replication for *E. coli* amplified from pUC19 (Gene Bank M77789). P*zeo* plasmid used for selection of double transformants consisted of two parts: (1) the *Sh ble* gene amplified from pUT737 (Cayla, Toulouse, France) placed under control of the P*pki* promoter and the T*cbh2* terminator, both amplified from pCAD_*mbfA*, and (2) origin of replication for *E. coli* linked to an ampicillin resistance cassette amplified from pUC19 (Gene Bank M77789). A schematic overview about the constructed cassettes can be found in Fig. S2 in the Supplementary Material. Cloning and isolation of the plasmids were performed according to standard methods (Sambrook and Russel, 2001). Before fungal transformation, bacterial elements were cut out with the *PmeI* restriction enzyme.

2.3. Transformation of A. niger

Protoplast transformation of *A. niger* was performed according to the previously described method (Blumhoff et al., 2013). The eGFP and CadA expression cassettes were transformed to the parental *A. niger* strain. Each of the aconitase expression cassettes (encoding ACO1, AcnA, AcoA) was co-transformed with a PCR product containing the CadA expression cassette amplified from pCAD_mbfA to the parental *A. niger* strain at a mass ratio of 1:5. The same aconitase cassettes were co-transformed with the pZeo plasmid to an *A. niger* mCadA clone at a mass ratio of 5:1. The DsRed cassette was co-transformed with the pZeo plasmid to *A. niger* mGFP_MTSicdA strain at 5:1 ratio. The mCadA and mAcoA expression cassettes were both co-transformed with the pZeo plasmid to the cCadA+cAcnA strain at a mass ratio of 5:5:1. The genotypes of the strains are listed in Table 1. All transformants were purified by single colony isolation on the selection medium at least once (Daud et al., 1985). The successful transformation of the expression cassettes was verified by PCR.

2.4. Preparation of cell-free extract

To obtain cell free extracts conidia were cultivated for 48 h at 33 °C (180 rpm) in baffled shake flasks on Vogel's medium. The harvested mycelia (~100 mg) were disrupted with a bead ruptor (five times, 20 s, 4 m/s) using acid washed glass beads with a diameter of 0.4–0.6 mm in the presence of 1 mL 0.2 M sodium phosphate buffer at pH 6.2 or pH 7.0 for CAD or ACO activity assay respectively. The protein concentration was determined using the Bio-Rad Protein Assay according to the manufacturer's instructions.

2.5. Measurement of enzyme activities

The *cis*-aconitate decarboxylase (CAD) activity and concentration of itaconic acid were determined according to Blumhoff et al. (2013). The reaction containing 8 mM freshly prepared *cis*-aconitic

Table 1
Strains of *A. niger* ATCC 1015 used in this study.

Strain name	Genotype			
	Promoter	MTS	Gene	Protein localization
cGFP	P*mbfA*	—	*eGFP*	cytosol
mGFP_MTSicdA	P*icdA*	MTS*icdA*	*eGFP*	mitochondrium
mGFP_MTSacoA	P*mbfA*	MTS*acoA*	*eGFP*	mitochondrium
mGFP_MTSicdA+mDsRed_MTSacoA	P*mbfA*	MTS*icdA*	*eGFP*	mitochondrium
	P*mbfA*	MTS*acoA*	*DsRed*	mitochondrium
cCadA	P*mbfA*	—	*cadA* of *A. terreus*	cytosol
cCadA+cACO1	P*mbfA*	—	*cadA* of *A. terreus*	cytosol
	P*mbfA*	—	*ACO1* of *S. cerevisiae*	cytosol
cCadA+cAcnA	P*mbfA*	—	*cadA* of *A. terreus*	cytosol
	P*mbfA*	—	*acnA* of *E. coli*	cytosol
cCadA+mAcoA	P*mbfA*	—	*cadA* of *A. terreus*	cytosol
	P*mbfA*	*MTSacoA*	*acoA* of *A. niger*	mitochondrium
mCadA	P*icdA*	MTS*icdA*	*cadA* of *A. terreus*	mitochondrium
mCadA+cACO1	P*icdA*	MTS*icdA*	*cadA* of *A. terreus*	mitochondrium
	P*mbfA*	—	*ACO1* of *S. cerevisiae*	cytosol
mCadA+cAcnA	P*icdA*	MTS*icdA*	*cadA* of *A. terreus*	mitochondrium
	P*mbfA*	—	*acnA* of *E. coli*	cytosol
mCadA+mAcoA	P*icdA*	MTS*icdA*	*cadA* of *A. terreus*	mitochondrium
	P*mbfA*	*MTSacoA*	*acoA* of *A. niger*	mitochondrium
cCadA +cAcnA+mCadA+mAcoA	P*mbfA*	—	*cadA* of *A. terreus*	cytosol
	P*icdA*	MTS*icdA*	*cadA* of *A. terreus*	mitochondrium
	P*mbfA*	—	*acnA* of *E. coli*	cytosol
	P*mbfA*	*MTSacoA*	*acoA* of *A. niger*	mitochondrium

MTS: Mitochondrial Targeting Sequence.

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 enzymatic formation of itaconic acid was followed with HPLC using a Rezex ROA column (300 × 7.8 mm) (Phenomemex, USA). The enzymatic reaction was stopped after 1 h by heat inactivation. One CAD unit is defined as the amount of enzyme that catalyzes the formation of 1 μM itaconic acid/min at 37 °C.

For the measurement of aconitase (ACO) activity, 200 μL of the cell-free extract was mixed with 30 mM of sodium citrate in 0.2 M sodium phosphate buffer (pH = 7.0). The reaction was carried out for 1 h at 37 °C and heat inactivated for 20 min at 80 °C. The ACO activity was determined by measuring the formation rate of isocitrate which was detected using the commercial kit Enzytec fluid for D-isocitric acid (R-Biopharm, Product Code E5220) following the manufacturer's instructions. One ACO unit is defined as the amount of enzyme that catalyzes the formation of 1 μM isocitrate/min at 37 °C.

2.6. Shake flask cultivation of A. niger

Conidia (10^7) were cultivated for 240–312 h at 33 °C on the shaker (180 rpm) in 1 L baffled shake flasks containing 100 mL Vogel's medium supplemented with 20% glucose and without $MnSO_4$. Determination of biomass content and concentration of glucose and organic acids were performed as previously described (Blumhoff et al., 2013). Each cultivation was done with at least three biological replicates.

2.7. Fluorescence microscopy

Conidia of the transformants expressing GFP and/or DsRed were incubated on microscope slides in 200 μL Vogel's medium at 30 °C for 27 h. Mitochondria were stained with 300 nM MitoTracker® Red CM-H_2XRos (Invitrogen, Cat. no. M7513), briefly washed with medium and analyzed under the Leica TCS SP5 II confocal laser scanning microscope using a 63 × /1.4 oil immersion objective. eGFP was exited with the 488 nm line of an Argon laser and was detected with a filter band of 500–550 nm. DsRed and MitoTracker Red were excited with the DPSS laser at 561 nm and detected with a filter band of 570–650 nm.

3. Results and discussion

3.1. Mitochondrial targeting

Itaconate is produced by decarboxylation of *cis*-aconitate, which is produced from citrate by dehydration. *A. niger* is the most efficient producer of citric acid. Based on this fact the general aim is to construct an efficient itaconic acid producer. Previously, we described the cytosolic expression of CadA in two different strains of *A. niger*: ATCC 1015 and CBS 513.88 (Blumhoff et al., 2013). We tested the strains on different media in order to find suitable conditions, where both citric acid and itaconic acid are produced at maximal titers. It was found that a modified Vogel's medium (lacking $MnSO_4$) was optimal to produce both acids. The overall titers of itaconic acid were higher in strains having a CBS 513.88 background. Maximal titers of 567 mg/L (on average 420 ± 140 mg/L) were obtained in strains with a CBS 513.88 background, whereas only maximal 120 mg/L (on average 54 ± 50 mg/L) were obtained in strains with an ATCC 1015 background. We have shown that the productivity correlates with the CadA protein abundance. Considering that CBS 513.88 is the more efficient protein producer and is the ancestor of industrially important enzyme production strains, it is not surprising that the achieved CadA abundance is higher in this strain background (Pel et al., 2007). However, strain ATCC 1015 is the predecessor of industrial citric acid production strains (Andersen et al., 2011) so we consider this genetic environment to be more suitable for the development of an acidogenic strain. This is why we started our present strain construction work in the background of ATCC 1015, despite the low titers accumulated by initial CadA expressing strains with this background.

As outlined above one crucial point for providing *A. niger* with CAD or additional ACO activity, is the decision in which

compartment the enzymes shall be located. Citric acid production and the following reactions of the TCA cycle take place in the mitochondrion. CadA catalyzing the decarboxylation of *cis*-aconitate is naturally localized in the cytosol. Aconitase exists in two forms—a mitochondrial as well as a cytosolic form. Overexpression of *cadA* in the cytosol of a wild type strain of *A. niger* led to the accumulation of about 54 ± 50 mg/L of itaconic acid. This value is comparable to recently published results of Li et al. (2013) where levels of about 50–135 mg/L itaconic acid were achieved with a *cadA* expressing strain. This very low titer of itaconic acid might be caused by substrate limitation. The exact concentration of citrate and *cis*-aconitate in mitochondria and the cytosol, respectively, are not amenable for measurement at this point. In order to increase the itaconic acid production, we attempted to overexpress *cadA* in the mitochondria and compare it to the cytosolic expression. To this end, we fused the *cadA* gene of *A. terreus* with two different mitochondrial targeting sequences (MTS) of *A. niger*: from isocitrate dehydrogenase (MTS*icdA*) and aconitase (MTS*acoA*), respectively. The genes were placed under the control of P*mbfA* or P*icdA*, both strong and constitutive endogenous promoters of *A. niger*. In order to verify the correct targeting of the heterologous proteins to the mitochondria by the chosen MTS, fusion proteins with both mitochondrial leader sequences replacing *cadA* with *eGFP* and *DsRed* were constructed. As a control, eGFP was expressed without any MTS leader. The constructs were integrated into the genome of *A. niger* ATCC 1015 and the protein localization was analyzed with fluorescence microscopy (Fig. 1).

As expected, eGFP without a leader sequence led to a homogenous distribution of eGFP in the entire cytosol (Fig. 1A). In contrast, the expression of eGFP with either of the predicted leader sequences led to a characteristic punctuate pattern of eGFP localization as can be expected from mitochondrial localization. Staining with MitoTracker, an agent which is used to specifically stain mitochondria (Poot et al., 1996; Helmerhorst et al., 1999) is consistent with the fact that eGFP is localized in the mitochondria (Fig. 1B+C). Co-expression of DsRed fused to MTS*acoA* with eGFP fused to MTS*icdA*, nicely shows a co-localization of both fluorescent proteins (Fig. 1D). This demonstrates that both MTS sequences can be used to target a heterologous protein to the mitochondria of *A. niger*.

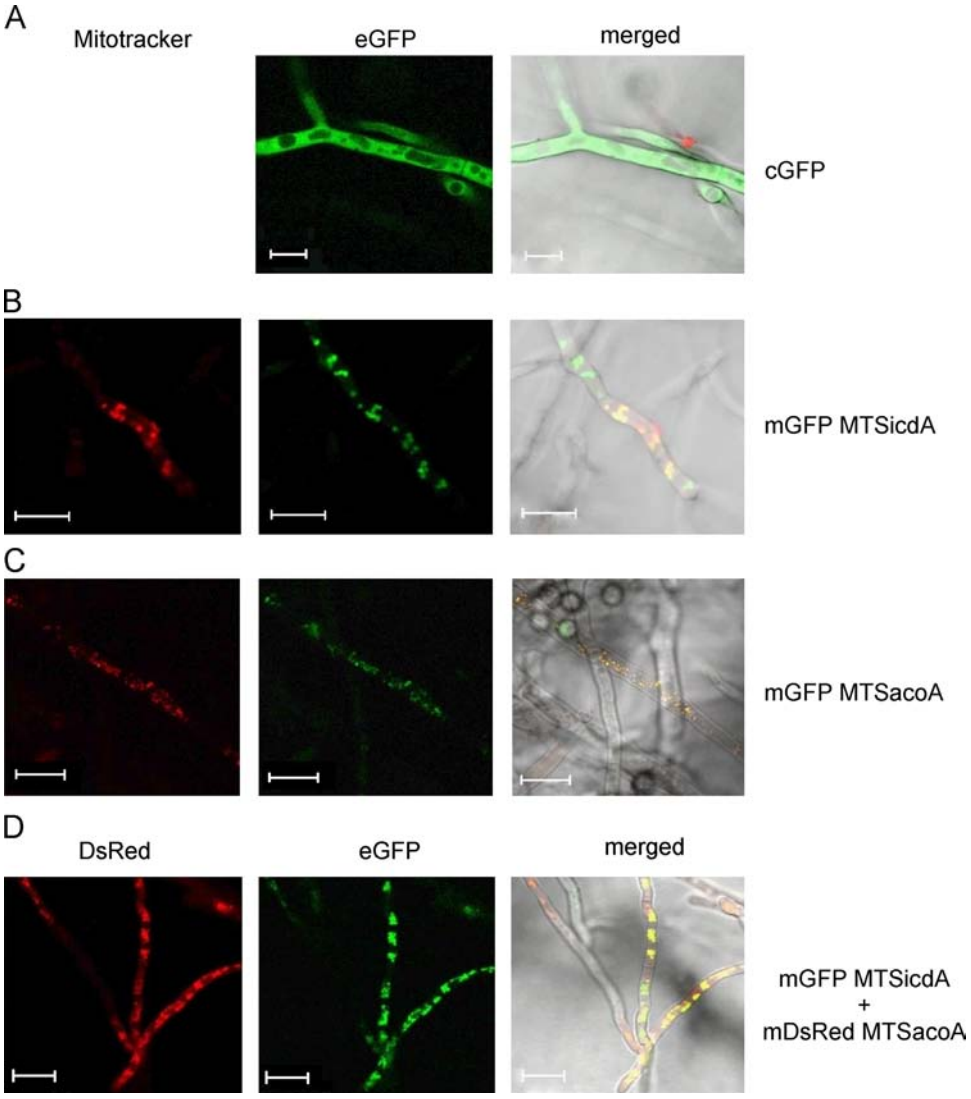

Fig. 1. Fluorescent images of *A. niger* strains containing different versions of fluorescent proteins targeted to the cytosol and the mitochondria using different mitochondrial targeting sequences (MTS). The fluorescent images (left side: red channel, middle: green channel) as well as the broad field image (right side) with the merged fluorescence signals are shown. eGFP was expressed with no MTS sequence leading to a cytosolic localization (A: cGFP). eGFP with an N-terminal fused MTS sequence of *icdA* or *acoA* gene can be detected in the mitochondria (B: mGFP MTSicdA; C:mGFP MTSacoA). For the visualization of mitochondria, hyphae were stained using MitoTracker® Red. Co-expression of DsRed with a MTS of *acoA* and eGFP with a MTS of *icdA* leads to a co-localization of both proteins in the mitochondria (D: mGFP MTSicdA+mDsRed MTSacoA). Scale bar: 10 μm.

3.2. Mitochondrial targeting of cis-aconitate decarboxylase (CAD)

After demonstrating that the correct localization of the heterologous protein can be obtained by fusion with one of these leader sequences, the *cadA* gene was mitochondrially expressed and the clones were analyzed for itaconic acid production in shake flasks. While strains expressing only cytosolic *cadA* accumulated on average 54 ± 49 mg/L of itaconic acid, the targeting of *cadA* into the mitochondrion resulted in a significantly improved level of 165 ± 90 mg/L itaconic acid on an average (Fig. 2).

We could show before that (at least in the present ranges of productivity) the itaconic acid production is directly correlated with the *cis*-aconitate decarboxylase (CAD) activity (Blumhoff et al., 2013). In order to exclude the possibility that a higher mitochondrial expression simply leads to higher CAD activities, the CAD activities were measured. In fact it turned out that the levels of the cytosolic CAD activity are slightly higher than those of the mitochondrial CAD (Fig. 2). However, the productivities of the strains with mitochondrially located CAD are significantly higher (2–3 fold), showing that proper localization is crucial for productivity.

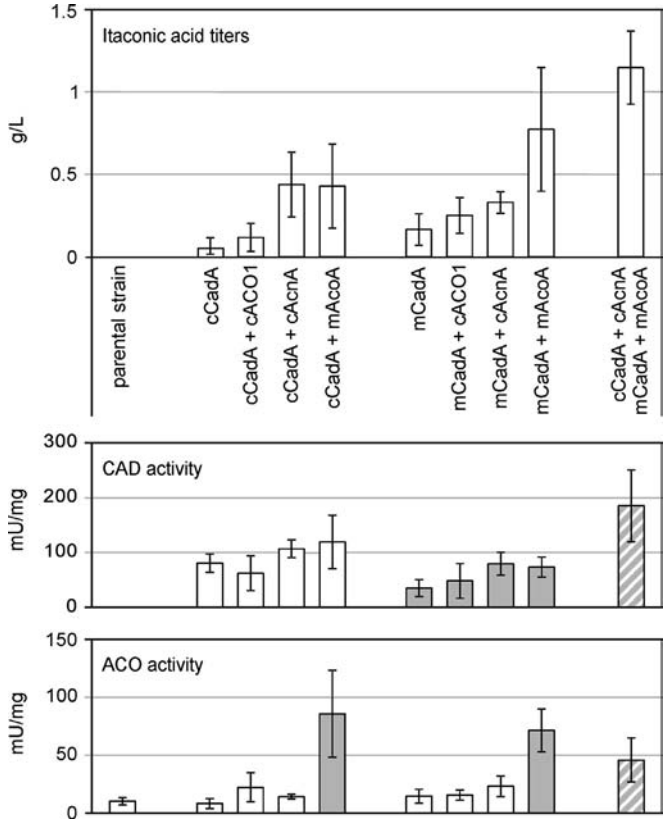

Fig. 2. Maximal itaconic acid titers, *cis*-aconitate decarboxylase (CAD activity) and aconitase (ACO activity) enzyme activities of different strains reached in the shake flask cultures. Gray bars show enzyme activities located in mitochondria. The following strains were measured: parental strain (ATCC 1015) and strains containing a cytosolic (cCadA) or mitochondrial (mCadA) *cis*-aconitate decarboxylase and a cytosolic or mitochondrial aconitase. Three different aconitases were tested: two cytosolic, one coming from *E. coli* (cAcnA) and the other from *S. cerevisiae* (cACO1). The mitochondrial aconitase was overexpressed from *A. niger* (mAcoA). Strains were grown in Vogel's medium for 48 h (CAD and ACO activity measurement) and for 240–312 h (itaconic acid titers). Itaconic acid titers in the culture supernatant [g/L] and activity values of cell free extracts [mU/mg protein] are the arithmetic mean of at least three different clones. Error bars show the standard deviation of the biological replicates.

3.3. Overexpression of aconitase in strains with CAD activity

In order to further increase the productivity of the strains we opted for overexpression of genes encoding different aconitases in order to increase the supply of the substrate for CAD. Three different aconitases were chosen: the bacterial *acnA* gene of *E. coli* and the *ACO1* gene of *S. cerevisiae* devoid of its native mitochondrial targeting signal, which are both localized in the cytosol and the mitochondrial *acoA* of *A. niger*. All aconitase genes were expressed with the *mbfA* promoter and co-transformed to *A. niger* ATCC 1015 together with the cytosolic or mitochondrial *cadA* gene.

3.3.1. Cytosolic aconitase expression

Co-expression of a cytosolic aconitase with cytosolic CadA leads to a significant increase of itaconic acid production compared to cytsolic CadA expression alone (Fig. 2). The rate limiting step for itaconic acid production under these conditions appears to be both the provision of *cis*-aconitate as a substrate for the CadA as well as the CAD activity itself. A direct comparison of the bacterial and the yeast aconitase revealed that the increase in ACO activity is similar with both constructs compared to the parental strain. However, the itaconate productivity was higher with the bacterial construct, which can be explained by the higher CAD activity in these strains.

Interestingly, co-expression of a mitochondrial aconitase leads to a similar itaconic acid productivity as observed with a cytosolic aconitase version pointing to a transport mechanism for *cis*-aconitic acid between the mitochondria and the cytosol. (Whose molecular basis is presently unknown.)

Co-expression of cytosolic aconitases with mitochondrial *cadA* also leads to increased itaconic acid production. However, the increases are lower compared to the cytosolic *cadA* clones (Fig. 2). The increase of the itaconic acid titer is not as pronounced as when the CadA protein is located in the same compartment. Again this points to the possibility of transport, which occurs from the cytosol to the mitochondria, or the mitochondrial export of *cis*-aconitate might be inhibited, by higher *cis*-aconitate concentrations in the cytosol, which in turn leads to higher *cis*-aconitate concentrations in the mitochondria.

3.3.2. Mitochondrial aconitase expression

A significant improvement is obtained when both enzymes, aconitase and *cis*-aconitate decarboxylase, are located in the mitochondria. With slightly lower enzyme activities compared to the cytosolic expression of both enzymes, higher itaconic acid productivities are achieved. One strain even achieved an itaconic acid titer of 1.2 g/L (on average 0.8 ± 0.4 g/L). Thus the improvement compared to the single cytosolic *cadA* expression is a factor of 14–24.

In a further experiment it was tested whether the combination of the cytosolic pathway and the mitochondrial pathway can further enhance the productivity (Fig. 3). For this purpose a cytosolic and a mitochondrial *cis*-aconitate decarboxylase as well as a cytosolic and mitochondrial aconitase were expressed together. Strains with this gene combination showed a clearly improved production of itaconic acid reaching with the best strain up to 1.4 g/L (on average 1.1 ± 0.3 g/L). Both enzyme activities are elevated in such strains but especially the *cis*-aconitate decarboxylase activity was significantly higher than in any other constructed strain.

These results demonstrate that the limiting factor for itaconic acid production is still to be found in the activity of the aconitase and *cis*-aconitate decarboxylase and in the localization of both enzymes in the same compartment. Furthermore, it can be concluded that the production limits of this fungus are not yet

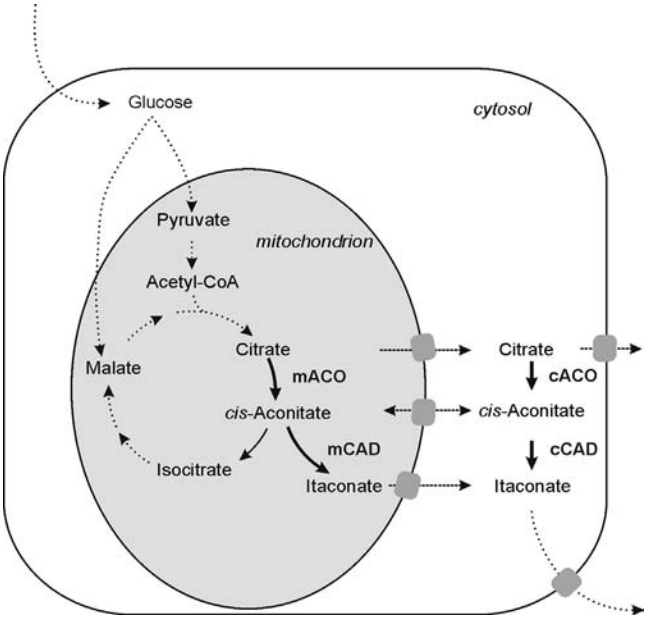

Fig. 3. Schematic drawing of metabolic pathways responsible for itaconic acid formation and their localization. Engineered reactions are highlighted with bold arrows. Mitochondrial and cytosolic aconitase (mACO/cACO) as well as mitochondrial or cytosolic *cis*-aconitate decarboxylase (mCAD/cCAD) are marked. Metabolite transporters are indicated by gray boxes. The occurrence of these proteins is speculative especially a transporter for itaconate and *cis*-aconitate. However, a citrate/malate antiporter is known to exist in *A. niger* (Pel et al., 2007), which might accept also other substrates.

reached although *A. niger* is in the metabolic state to produce organic acids under the applied conditions. The major acid produced is citric acid with levels ranging between 25 and 40 g/L (data not shown). Therefore it is not surprising that no correlation between citric acid and itaconic acid formation can be observed in the analyzed cultures because the itaconic acid titers reach only 3–7% of the secreted organic acids.

The *cis*-aconitate decarboxylase levels with about 185 ± 66 mU/mg are still 5 times lower compared to the levels reached with strain CBS 513.88 (1058 ± 570 mU/mg). As it was found that the CadA protein is rather unstable *in vitro* (Dwiarti et al., 2002; Kanamasa et al., 2008) its stability might be also low *in vivo*. Therefore, it needs to be checked whether the expression levels can be still increased by the introduction of further gene copies or if the stability of the CadA gene can be increased by a protein engineering strategy. Another point of interest in this context regards the liberation of *cis*-aconitate from aconitase. Aconitase catalyzes both, the dehydration of citrate or isocitrate to *cis*-aconitate as well as the hydration of *cis*-aconitate to citrate or isocitrate. Generally, the concentrations in a mixture are citrate$\gg$isocitrate $>$ *cis*-aconitate (Schomburg et al., 2013). Enzyme engineering to change the kinetics of this reaction favoring the formation of *cis*-aconitate might bring a major step forward for itaconic acid production. A further target for strain engineering is centered around transport mechanisms. Due to the compartmentalization metabolites need to be transported within the cell and out of the cell.

4. Conclusions

Many metabolic pathways are distributed over various cellular organelles in eukaryotic cells. Therefore, substrate availability differs significantly within a cell. This includes carbon compounds as direct substrates but also co-factors. Consequently, transport processes play generally a crucial role, when analyzing and attempting to modify metabolic pathways. However, also the targeting of enzymes to the correct compartment is an important means for metabolic engineering. The metabolic precursors for itaconic acid, namely citrate and *cis*-aconitate are naturally synthesized in the mitochondria. In order to analyze the effect of compartmentalization, aconitase and *cis*-aconiate decarboxylase are expressed in this work either in the cytosol or the mitochondria. We show for the first time the mitochondrial targeting of a heterologous production pathway in *A. niger*. Heterologous expression of *cis*-aconitate decarboxylase and aconitase in the mitochondria of *A. niger* doubles the productivity compared to strains which express both enzymes in the cytosol. A combination of the cytosolic and the mitochondrial pathway in one strain leads to an even higher itaconic acid production.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.05.003.

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Autonomously replicating plasmids as a transient expression tool in *Aspergillus niger*

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Abstract

Nowadays, genetic engineering plays a great role for the improvement of the industrial *Aspergillus niger* cell factories. Advanced genetic strategies call for efficient genetic tools that enable various and complex genetic modifications and allow for quick and effective transformation methods. The possibility of having well-characterized autonomously replicating plasmids for filamentous fungi broadens the horizon of genetic engineering. So far, the only known fungal origin of replication AMA1 causes many difficulties during plasmid construction due to its long size and palindromic structure. Our effort was to shorten the AMA1 fragment and verify its replicating properties and stability. We demonstrated that only one of the two palindromic sequences together with the central region of AMA1 is required to maintain autonomous replication in *A. niger*. Such a modification of AMA1 did not influence the transformation efficiency. However, further shortening caused a drastic decline of the transformation efficiency. Furthermore, we demonstrated that the plasmid containing "one-arm" AMA1 was removed from cells after at the latest in the 3rd generation when spores were cultivated without antibiotic pressure. Such a system represents a powerful transient expression tool for genetic engineering of *A. niger*.

1. Introduction

Aspergillus niger is a widely utilized organism for the production of food processing enzymes and metabolites such as organic acids or antibiotics on large industrial scale (Sauer et al. 2008; Tevz et al. 2010; Dashtban et al. 2011; Frisvad et al. 2011). Over the recent years the rational engineering of industrial strains has become an important issue for strain improvement. The methods and technologies for genetic engineering of *A. niger* have rapidly evolved. Consequently, advanced techniques and developments demand for genetic tools that enable various and complex genetic modifications. The efficient genetic tool-box should allow for quick and effective transformation methods and screening of transformants.

One important tool is autonomously replicating plasmids. Genetic manipulation of fungi using autonomous replicating plasmids has advantages over the integrative system because of the high-frequency of transformation achieved (Gems et al., 1991; Aleksenko and Clutterbuck, 1996, 1997). Moreover, it allows recovery of insert-containing plasmids for further genetic analyzes. In *Aspergillus*, similar to most of filamentous fungi, homologous recombination occurs at very low frequencies due to ectopic integration of the transforming DNA. In some cases, it is preferred to express genes extrachromosomally to avoid random integration and unknown mutations in the genome. In addition, the autonomous plasmids could be used to enhance the frequency of homologous recombination by increasing the time of plasmid presence in the cell.

Nevertheless, it is important to know how long introduced plasmids remain in the cell after transformation. Plasmids that are stable only under selective conditions are a useful tool for genetic engineering. Such extrachromosomal plasmids can be used as transient vectors to carry genes that should be expressed only for limited period of time. Temporary expression of the genes may be useful for proteins that act as activators of certain processes

or are required only for single action. Tools like recombinases or reporter genes can easily be inserted to cells and just as well be expelled when taking away the selective pressure e.g. antibiotic addition. Furthermore, co-transformation with transient autonomously replicating selectable plasmids enables a recovery of the marker. This is particularly important when multiple strain transformations are required e.g. for expression of subsequent genes of the entire gene clusters or certain enzymes of metabolic pathways.

Nowadays, origins of replication for bacterial and yeast systems are very well investigated. Many bacteria and yeasts contain naturally occurring plasmids that contain an origin of replication. An autonomously replicating sequence (ARS) of the yeasts genome initiates a replication event when cloned into a plasmid (Newlon and Theis 1993). Autonomously replicating vectors for these organisms show very high transformation frequencies. Unfavorably, in contrast to yeasts and bacteria, filamentous fungi lack natural extrachromosomally replicating DNA elements. Nevertheless, it is possible to isolate from genomes of some filamentous fungi DNA fragments which can provide extrachromosomal maintenance of plasmids (Aleksenko and Clutterbuck 1997). For a long time none of the fungal sequence was known to be responsible for this property. In 1991, Gems et al. isolated from *A. nidulans* a 6.1 kb DNA fragment that promotes autonomous plasmid replication in fungi. This fragment, designated AMA1 was proven to be an effective plasmid replicator. It has been demonstrated that AMA1 strongly enhances the transformation efficiency of *A. nidulans* up to 10^5 transformants per µg DNA (Gems et al. 1991). Furthermore, an AMA1 bearing plasmid enables plasmid replication without its rearrangement, multimerization or integration into the host genome (Aleksenko and Clutterbuck 1997). In today's biotechnological applications plasmids containing the AMA1 region are frequently used for the transformation of filamentous fungi. AMA1 has been described as a functional origin of

replication in *A. nidulans*, *A. niger*, *Giberella fujikuroi* and *Penicillium chrysogenum* (Fincham 1989; Fierro et al., 1996).

AMA1 remains a challenge with regard to vector construction and design due to its big size and palindromic structure. It consists of a short 0.3 kb central region flanked by two inverted almost 3 kb long repeats. These two MATE1 elements act as mobile transformation enhancers (Aleksenko and Clutterbuck 1997). Such a structure of AMA1 fragment creates many difficulties during genetic manipulations e.g. the PCR amplification. In this study we modified the AMA1 fragment in order to provide a simple, effective and robust genetic tool. The shortened versions of AMA1 were tested for their replicating properties and stability.

2. Materials and Methods

2.1. Strains and media

Escherichia coli TOP10 served as host for recombinant DNA manipulation. *A. niger* ATCC 1015 was used as parental strain for *A. niger* transformation. *E. coli* transformants were cultivated on LB (Sambrook and Russell 2001) supplemented with 100 µg/mL ampicillin. *A. niger* was cultured in liquid ME or solid MEA (Blumhoff et al., 2013) supplemented with 150 µg/mL hygromycin B.

2.2. Contruction of expression cassettes

Plasmids were constructed using an enzymatic assembly method (Gibson et al., 2009). Target DNA fragments were obtained as previously described (Blumhoff et al., 2013). The primers used for PCR amplification are specified in the Table 1. Expression cassettes containing original or modified AMA1 fragment were composed of four fragments: (1) the origin of replication for *E. coli* and ampicillin resistance cassette amplified from pUC19 (Gen-

Bank Accession No. M77789); (2) the *gusA* coding sequence of *E. coli* amplified from genomic DNA of strain TOP10 under control of *gpdA* promoter amplified from pAN7-1 (GeneBank Accession No. Z32698.1) and *TrpC* terminator amplified from pAN52-1Not (GeneBank Accession No. Z32524); (3) the *hph* gene providing hygromycin B resistance of fungi amplified from pAN7-1 under control of P*tr1* promoter amplified from pUT737 (Cayla) and T*niaD* terminator amplified from genomic DNA of *A. nidulans*; and (4) the full-size or shortened AMA1 fungal origin of replication amplified from pRG3-AMA1-NotI (Osherov et al. 2000) kindly provided by Joseph Strauss, University of Natural Resources and Life Sciences, Vienna (BOKU). The modified AMA1 fragments contained the central region and full-size (AMA1/2) or shortened (AMA2-AMA6) left palindromic sequence. The PCR products of shortened AMA1 fragments had the following sizes: AMA1/2: 2877 bp, AMA2: 2587 bp, AMA3: 2354 bp, AMA4: 2075 bp, AMA5: 1827 bp, AMA6: 1588 bp. Cloning and isolation of the plasmids were performed according to standard methods (Sambrook and Russel, 2001).

2.3. Transformation of *A. niger*

Transformation of *A. niger* was performed according to the previously described method based on transformation of protoplasts in the presence of calcium ions and PEG (Blumhoff et al., 2013). However, the transformation 2 (low efficiency) slightly differed from this protocol due to not optimized transformation method at this time point. In each transformation, 5.36 µg DNA was transformed to at least 10^8 protoplasts.

2.4. Stability experiment

Fungal colonies were picked from transformation plates and grown on MEA supplemented with hygromycin until conidia were formed. Conidia were harvested and

filtered through Miracloth (Calbiochem). Defined amount of conidia (50-1000 conidia in total) was plated on MEA with and without hygromycin. The percentage of AMA containing conidia was determined by counting the number of arising colonies on both plates. Conidia were harvested and transferred to another selective and non-selective plate.

Table 1. Primers used in the PCR of the plasmid fragments

Primer	Sequence
ori_AmpR_AE_FW	CCTAGGCTAGCTCTAGACCACACGTTTTCCATAGGCTCCGCCCCCCTGAC
ori_AmpR_AE_BW	GCCTGTGTGTAGAGATACAAGGGAATTCATGAGTATTCAACATTTCCGTGTCG
gusA_AE_FW	CGACACGGAAATGTTGAATACTCATGAATTCCCTTGTATCTCTACACACAGGC
gusA_AE_BW	CCATATGGTGCACTCTCAGTACAATCGAGTGGAGATGTGGAGTGGGCGCTT
HygB_AE_FW	AAGCGCCCACTCCACATCTCCACTCGATTGTACTGAGAGTGCACCATATGG
HygB_AE_BW	CCGATACGTAACGCGTCTGCAGCATCTCTAGTCTACAGTGGCCGCCTTGT
AMA1_AE_FW	ACAAGGCGGCCACTGTAGACTAGAGATGCTGCAGACGCGTTACGTATCGG
AMA1_AE_BW	GTCAGGGGGGCGGAGCCTATGGAAAACGTGTGGTCTAGAGCTAGCCTAGG
AMA_2_(HgmAE)_FW	ACAAGGCGGCCACTGTAGACTAGAGGGTTTGCGTTAAACTAAATCAGAGCCCG
AMA_3_(HgmAE)_FW	ACAAGGCGGCCACTGTAGACTAGAGCCAGGTTAGTTGCAACTAATACTAGTTAG
AMA_4_(HgmAE)_FW	ACAAGGCGGCCACTGTAGACTAGAGCCAGAACCACAAAGGAGAAATGTCTTACC
AMA_5_(HgmAE)_FW	ACAAGGCGGCCACTGTAGACTAGAGCCTCGTCAATCGATGAGAAAAAGCGCC
AMA_6_(HgmAE)_FW	ACAAGGCGGCCACTGTAGACTAGAGGGTACGGGGCTGAATGTAACTTGCCTTTCC
HgmAE_(AMA2)_BW	CGGGCTCTGATTTAGTTTAACGCAAACCCTCTAGTCTACAGTGGCCGCCTTGT
HgmAE_(AMA3)_BW	CTAACTAGTATTAGTTGCAACTAACCTGGCTCTAGTCTACAGTGGCCGCCTTGT
HgmAE_(AMA4)_BW	GGTAAGACATTTCTCCTTTGTGGTTCTGGCTCTAGTCTACAGTGGCCGCCTTGT
HgmAE_(AMA5)_BW	GGCGCTTTTTCTCATCGATTGACGAGGCTCTAGTCTACAGTGGCCGCCTTGT
HgmAE_(AMA6)_BW	GGAAAGGCAAGTTACATTCAGCCCCGTACCCTCTAGTCTACAGTGGCCGCCTTGT
AMA_short_(Amp)_BW	GTCAGGGGGGCGGAGCCTATGGAAAGGAGTTATCTGCGACCACTGGACTCTC
Amp_(AMA_short)_FW	GAGAGTCCAGTGGTCGCAGATAACTCCTTTCCATAGGCTCCGCCCCCCTGAC

3. Results and Discussion

3.1. Shortening and functionality of AMA1 plasmids

We constructed six plasmids bearing shortened AMA1 fragments. Each of the shortened AMA1 contained the central region and only one of two palindromic sequences. The remaining "one-arm" contained the full sequence (AMA1/2) or was further shortened (AMA2-AMA6). The sizes of modified AMA1 sequences varied from 1588 bp to 2877 bp. Shortened "one-arm" AMA1 fragments as well as full-size AMA1 were assembled into plasmids with the GUS reporter gene and a hygromycin resistance cassette. Plasmids were transformed into the wild-type strain of *A. niger* ATCC 1015 in two independent experiments with the same total amount of DNA (5.36 µg) (Fig. 1). The total transformation efficiency was higher in the first experiment, in which the optimized transformation protocol was applied. Both experiments showed high transformation efficiencies, when full-size AMA1 and "one-arm" fragment (AMA1/2) were supplied. In the transformation with the full-size AMA1, 260 colonies per µg DNA were obtained. The transformation efficiency only slightly decreased in case of "one-arm" constructs (200 colonies per µg DNA). Further shortening (AMA2, AMA3) led to a drastic decline of the transformation efficiency. Nevertheless, 20-30 transformants per µg DNA were still obtained. The transformation of even shorter fragments (AMA4, AMA5 and AMA6) did not yield any transformants. This data demonstrates that only one of the two palindromic sequences together with the central region of AMA1 are required to maintain autonomous replication in *A. niger*. The full-size of the remaining "arm" is necessary to provide high-transformation efficiency. The shortening up to 2354 bp results in the elimination of the sequence responsible for transformation efficiency, but not the replication properties. This data proves that the problematic full-size AMA1 fragment can be

successfully replaced by the "one-arm" AMA1 in genetic manipulations. The removal of one of the two palindromic sequences simplifies the use of this fragment in genetic manipulations and enables quick and robust vector construction.

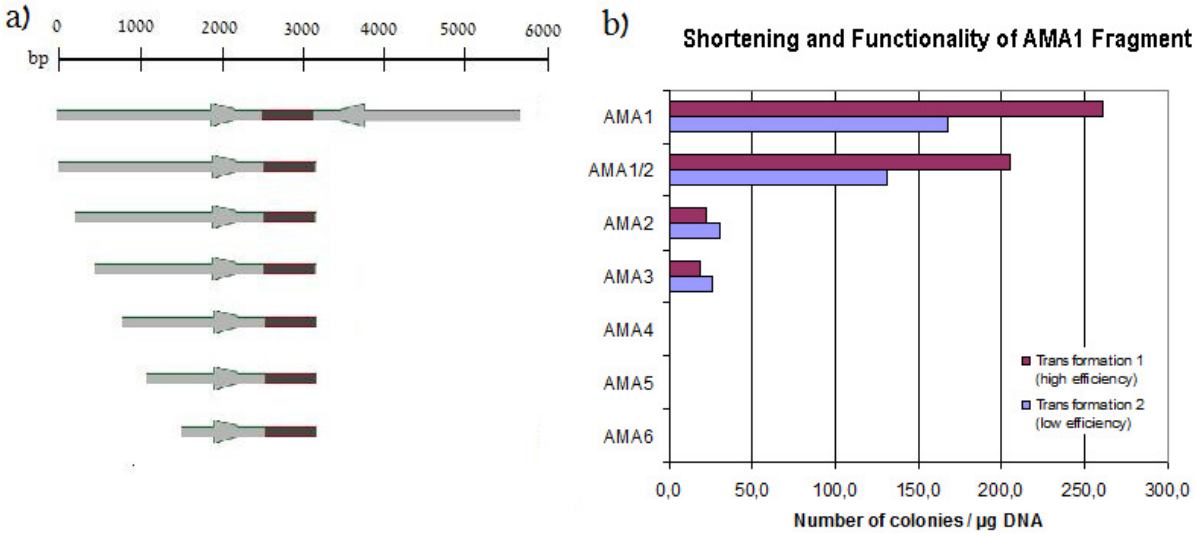

Figure 1. Original and shortened from 2877 bp to 1588 bp AMA1 fragments (a) used for *A. niger* ATCC 1015 transformation during two independent experiments (b).

3.2. Plasmid stability

The stability of the autonomously replicating plasmids is an important issue in genetic engineering. Both, stable and transient plasmids can be a useful genetic tool for various applications. The plasmid stability can be sustained by continuous selection pressure e.g. antibiotic addition. We investigated the stability of three different AMA1 constructs (AMA1, AMA1/2, AMA2) under both selective and non-selective conditions. Three clones of each construct were analyzed for three rounds of plating on medium with and without hygromycin (Fig.2). The percentage of plasmid containing conidia was determined by plating a defined amount of conidia on both selective and non-selective plates and counting the number of arising colonies (Fig.3).

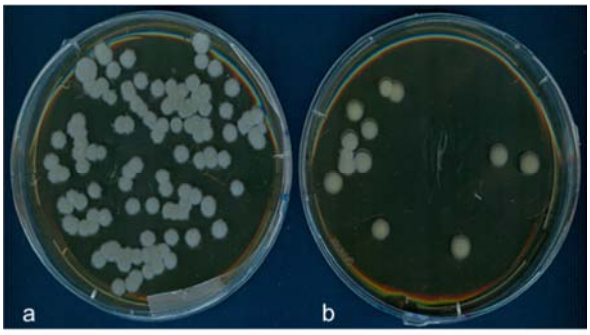

Figure 2. Colonies from spores plated on non-selective (a) and selective (b) conditions.

The experiment showed that almost all the plasmids were lost at the latest in the 3rd generation when the spores were cultivated without antibiotic pressure. The plasmids were more stable when hygromycin was added to the plates. Interestingly, in some clones the percentage of AMA1 containing spores was significantly increasing (up to 100 %) after following rounds of plating. This occurrence did not appear, when no selection pressure was applied. These results suggest that the plasmids could be integrated into genome if antibiotic pressure is sustained.

The plasmid containing the full-size AMA1 appeared to be more stable than these with the shortened fragments. It makes the shortened AMA1 fragment a powerful genetic tool. “One-arm” AMA1 combines the positive effects of high transformation efficiency with the possibility to remove the plasmid from the cells by growing the fungus under non-selective conditions. Such transient vectors can be applied for various genetic engineering purposes, where temporary expression of the genes is required.

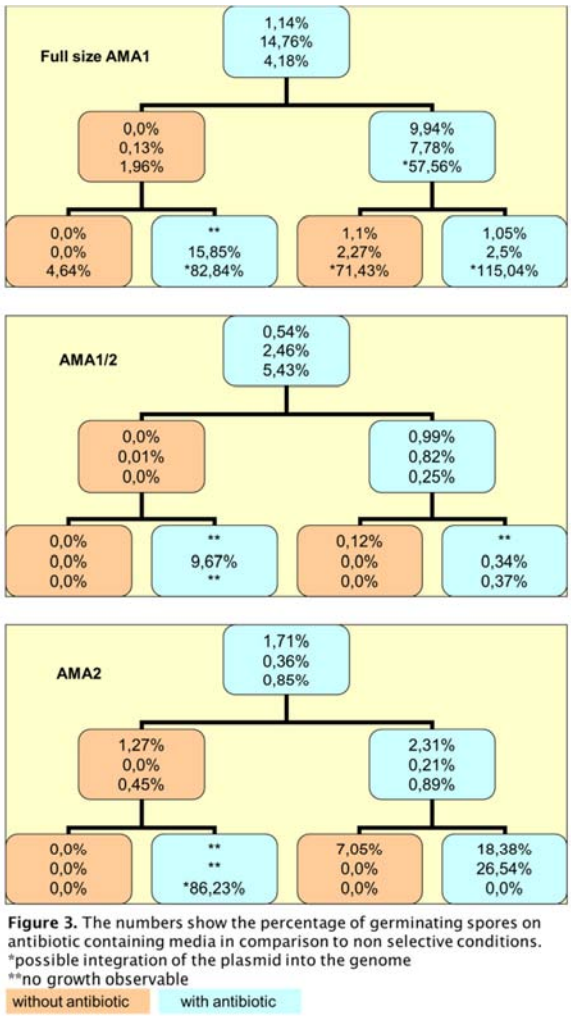

Figure 3. The numbers show the percentage of germinating spores on antibiotic containing media in comparison to non selective conditions.
*possible integration of the plasmid into the genome
**no growth observable

4. Conclusion

In this study, we modified an autonomously replicating element for filamentous fungi and proved its functionality as fungal origin of replication in *A. niger*. We demonstrated that "one-arm" AMA1 fragment maintains its replicating properties without affecting transformation efficiency. In contrast to full-size AMA1, the modified fragment enables quick and simple construction of self-replicating expression cassettes. It can serve as genetic tool for construction of transient vectors for temporary gene expression. This genetic engineering strategy for *A. niger* can be beneficial to the ongoing work with industrial fungal systems.

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Tool box for the transformation of industrial filamentous fungi

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ABSTRACT:
Nowadays, genetic engineering plays a great role for the development of new industrial bioprocesses, where microorganisms are used for production of proteins and chemicals. However, rational engineering of highly selected strains is significantly limited because often resistance to genetic modification is observed and appropriate methods are needed. This *FHplus* project aims at the implementation of genetic tools for industrial filamentous fungi as a starting point for metabolic engineering. We succeeded to optimise the transformation protocol for lab and industrial strains of *A. niger* reaching up to 260 transformants per µg DNA. We developed autonomously replicating plasmids containing modified AMA1 fragments as the origin of replication for fungi and applied an innovative method for the plasmid construction. Additionally, we focused on quick, simple and efficient screening methods for transformants by using an improved GUS reporter system. It leads to intensive colour of the fungal colonies, which is directly visible on the plates and allows an estimation of the gene expression level.

INTRODUCTION

Microorganisms are commonly used for the production of proteins or chemicals in large industrial scale. Industrial strains frequently used for this purpose have the ability to over-produce the desired substances in comparison to wild type strains. Genetically modified microorganisms can express and produce heterologous proteins. Furthermore, metabolic pathways can be altered by metabolic engineering and enable the economical production of chemicals. One important biotechnological product is citric acid, which is frequently used in food industry and produced by the filamentous fungus *A. niger*. The current production process of citric acid has been established by mutagenesis and screening of wild type strains. Rational engineering did not take place due to the lack of appropriate methods. Because of the mutagenesis approach, all mutations of the production strains are unknown. These strains behave very different than the parental strain

and are very resistant to genetic manipulations, rendering them recalcitrant to metabolic engineering, which requires a large and efficient set of engineering tools.

TOOL 1: FUNGAL TRANSFORMATION

One of the most important steps of genetic engineering is to have an efficient transformation protocol at hand. Since production strains are very recalcitrant to genetic modifications, the protocol should provide high number of transformants. We succeeded to optimise the transformation protocol based on available methods [1], which does not only work with wild type strains of *A. niger*, but also with the industrial strains. The efficiency reaches up to 260 transformants per µg DNA, when an autonomously replicating plasmid is transformed.

TOOL 2: AUTONOMOUSLY REPLICATING PLASMIDS

We succeeded to implement an innovative way of plasmid construction developed by Gibson *et al*. in 2009 [2], based on an enzymatic assembly of overlapping PCR fragments. Thanks to this method many different expression cassettes can be created within a few days with an overall efficiency of 95% positive clones.

Autonomously replicating plasmids are not common for filamentous fungi, because of a lack of sequences enabling the initiation necessary for the replication. In 1991, Gems *et al*. isolated a 6.1 kb DNA fragment strongly enhancing the transformation efficiency and which enables to maintain extrachromosomal replication [3]. This AMA1 sequence consists of a 0.3 kb central region flanked by two palindromic sequences. However, inverted, almost 3 kb long repeats cause difficulties during plasmid construction e.g. during the PCR amplification. We investigated shortened AMA1 fragments devoiding one of the two palindromic sequences and their influence on the transformation efficiency. Shortened "one-arm" AMA1 fragments as well as full-size AMA1 were assembled into plasmids and transformed into the wild type strain of *A. niger* CBS 113.46.

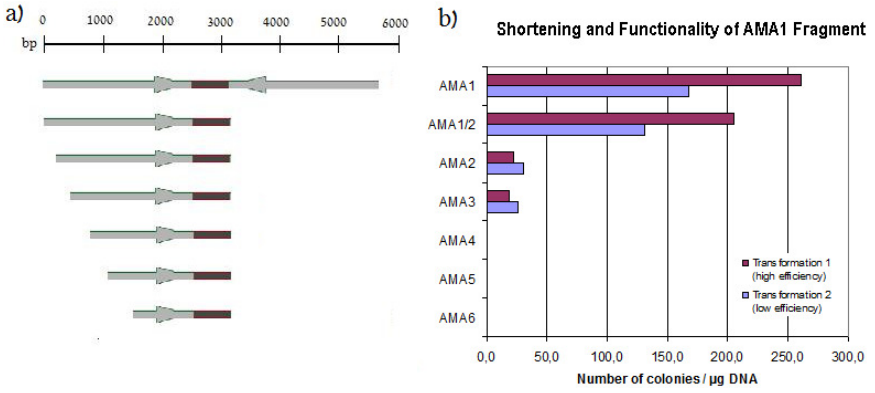

Figure 1. Original and shortened from 200 to 1000 bp AMA1 fragments (a) used for *A. niger* CBS 113.46 transformation during two independent experiments (b).

We performed two independent experiments transforming a total amount of 5.36 µg DNA (Fig. 1). Both experiments showed high transformation efficiencies, when full-size AMA1 and "one-arm" devoided fragment (AMA1/2) were supplied. The efficiency slightly decreased in case "one-arm" of the palindromic sequence was used. This demonstrates that only one of the two palindromic sequences together with the central region of AMA1 are required to maintain autonomous replication in *Aspergillus niger*. Further shortening (AMA2, AMA3) caused a drastic decline of the transformation efficiency. However, 20-30 transformants per µg DNA were still obtained. The even shorter fragments AMA4, AMA5 and AMA6 did not yield any transformants.

TOOL 3: GUS REPORTER SYSTEM

Quick and simple screening of transformants requires an efficient reporter. A GUS reporter system lead to intensive colour formation of the fungal colonies, which can be directly seen on the plates. We constructed expression cassettes containing the β-glucuronidase gene (*gusA*) from *E. coli* under the strong constitutive fungal *gpdA* promoter. X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) added to the transformation selective plates can be used as a substrate by the synthesized GUS protein, which leads to a blue colour formation. Positive blue fungal colonies can be easily selected among the other white colonies (Fig. 2.).

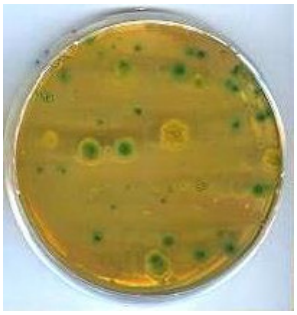

Figure 2. Selection plate after *Aspergillus niger* CBS 113.46 transformation with plasmid containing AMA1. Blue colonies correspond to GUS activity.

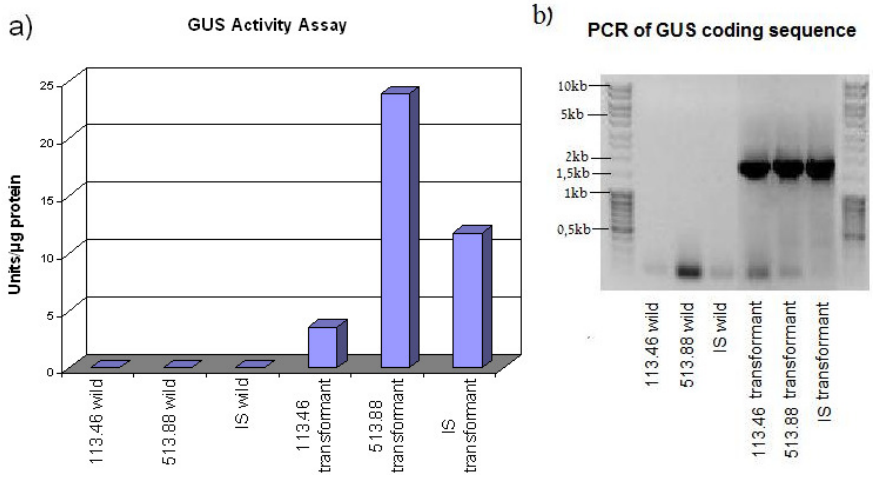

Figure 3. (a) β-glucuronidase activity assay performed using two wild type strains (CBS 113.46, CBS 513.88) and the industrial strain (IS) of *Aspergillus niger*, non-transformed and transformed strains with plasmid containing *gpd*AGUS gene and AMA1/2. (b) PCR of *gusA* gene from genomic DNA of the same strains (product size 1808bp).

Using the GUS system, the expression levels can be measured making use of a sensitive fluorometric assay. This assay is based on the reaction of 4-methylumbelliferyl-beta-D-glucuronide (MUG) catalyzed by β-glucuronidase, which results in a fluorescent end-product. With this assay very low concentrations of the enzymatic activity can be measured, which makes this method very powerful and useful for genetic engineering purposes. We performed GUS activity assays with two non-transformed wild type strains and an industrial strain of *A. niger* and with the same strains transformed with the plasmid containing the GUS gene and AMA1/2 sequence.

All three transformants show very high enzyme activity in contrast to their non-transformed equivalents (Fig. 3a). This result was confirmed by PCR. The amplification of the GUS coding sequence from genomic DNA of transformed and non-transformed strains gave only PCR products in case of transformant strains (Fig. 3b).

TOOL 4: SELECTION MARKER SHUTTLE VECTOR

In order to create an efficient shuttle vector for *E. coli* and filamentous fungi we made use of the *pki* promoter of *Trichoderma reesei.* Recently, it was shown that this promoter functions not only in *T. reesei* but also *E. coli* [4]. We constructed plasmids, in which the *pki* promoter was placed in front of the hygromycin resistance marker. We succeeded in transformation of *E. coli* as well as *A. niger* and successfully selected them on hygromycin. The promoter shows a strong constitutive expression in the fungal host and a weaker expression in the bacterial system.

CONCLUSION

The presented tools provide an efficient set for genetic engineering of filamentous fungi. We improved the transformation method, which allows now both the transformation of wild type strains as well as of an industrial *A. niger* strain. We monitored the transformation by using the effective GUS reporter system, which allowed to screen positive blue colonies directly on selective plates and to quantitatively measure the expression with a fluorometric assay. Additionally, we proofed the functionality of a truncated AMA1 sequence as a fungal origin of replication. Finally, we studied a shuttle vector system, which works both in the fungal and the bacterial system. All in all, we established efficient strategies for genetic engineering, which can be beneficial to the ongoing work with industrial fungal systems.

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Biochemistry of microbial itaconic acid production

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Itaconic acid is an unsaturated dicarbonic acid which has a high potential as a biochemical building block, because it can be used as a monomer for the production of a plethora of products including resins, plastics, paints, and synthetic fibers. Some *Aspergillus* species, like *A. itaconicus* and *A. terreus*, show the ability to synthesize this organic acid and *A. terreus* can secrete significant amounts to the media (>80 g/L). However, compared with the citric acid production process (titers >200 g/L) the achieved titers are still low and the overall process is expensive because purified substrates are required for optimal productivity. Itaconate is formed by the enzymatic activity of a *cis*-aconitate decarboxylase (CadA) encoded by the *cadA* gene in *A. terreus*. Cloning of the *cadA* gene into the citric acid producing fungus *A. niger* showed that it is possible to produce itaconic acid also in a different host organism. This review will describe the current status and recent advances in the understanding of the molecular processes leading to the biotechnological production of itaconic acid.

Keywords: *cis*-aconitic acid decarboxylase, *Aspergillus terreus*, *Aspergillus niger*, metabolic engineering, biochemical pathways, microbial organic acid production, industrial microbiology

INTRODUCTION

Itaconic acid (2-methylidenebutanedioic acid) is an unsaturated di-carbonic acid. It has a broad application spectrum in the industrial production of resins and is used as a building block for acrylic plastics, acrylate latexes, super-absorbents, and anti-scaling agents (Willke and Vorlop, 2001; Okabe et al., 2009). Since the 1960s the production of itaconic acid is achieved by the fermentation with *Aspergillus terreus* on sugar containing media (Willke and Vorlop, 2001). Although also other microorganisms like *Ustilago zeae* (Haskins et al., 1955), *U. maydis*, *Candida* sp. (Tabuchi et al., 1981), and *Rhodotorula* sp. (Kawamura et al., 1981) were found to produce itaconic acid, *A. terreus* is still the dominant production host, because so far only bred strains of this species can reach levels of up to 80–86 g/L (Okabe et al., 2009; Kuenz et al., 2012). Since the 1990s, itaconic acid as a renewable material is attracting a lot of interest. Although the production costs for itaconic acid are declining in the last years ($ 4 per kg in 2001; Willke and Vorlop, 2001), it is still a valuable product with an estimated price of $ 2 per kg. Currently, the worldwide production capacity of itaconic acid is expected to be about 50 kt per year, facing a demand of about 30 kt (Shaw, 2013, Itaconix Corporation, personal communication). Especially, for the production of polymers it is of interest, because in the future it can function as a substitute for acrylic and methacrylic acid used for the production of plastics (Okabe et al., 2009). However, these applications require an even lower price of the starting material. The current knowledge about the biotechnological production of itaconic acid was recently reviewed (Willke and Vorlop, 2001; Okabe et al., 2009). The latter review covers the industrial production of itaconic acid and the applications of this product. Therefore, we focus in this report on the recent advances with an emphasis on the biochemistry of the process and new genetic engineering targets. For rational strain improvement, it is essential to understand the underlying biological concepts and biochemical pathways leading to the production of this important organic acid in microorganisms.

BIOSYNTHESIS PATHWAY

Kinoshita (1932) recognized that a filamentous fungus was able to produce itaconic acid and consequently described this species as *A. itaconicus*. The biosynthesis of itaconic acid was for a long time hotly debated, because it was not clear whether itaconic acid arises from a pathway including parts of the tricarboxylic acid (TCA) cycle or an alternative pathway via citramalate or the condensation of acetyl-CoA.

Bentley and Thiessen (1957a) proposed a pathway for the biosynthesis of itaconic acid, which is depicted in **Figure 1**. Starting from a sugar substrate like glucose the carbon molecules are processed via glycolysis to pyruvate. Then the pathway is split and part of the carbon is metabolized to Acetyl-CoA releasing a carbon dioxide molecule. The other part is converted to oxaloacetate so that the previously released carbon dioxide molecule is again incorporated. In the first steps of the citric acid cycle, citrate and *cis*-aconitate are formed. In the last step, the only itaconic acid pathway dedicated step, *cis*-aconitate decarboxylase (CadA) forms itaconic acid releasing carbon dioxide. This pathway was confirmed by tracer experiments with ^{14}C and ^{13}C labeled substrates (Bentley and Thiessen, 1957a; Winskill, 1983; Bonnarme et al., 1995) and also the necessary enzymatic activities have been all determined (Jaklitsch et al., 1991).

The formation of carboxylic acids, like citric and itaconic acid, involves the shuttling of intermediate metabolites between different intracellular compartments and utilizes the different enzymatic capabilities of the respective compartment. In case

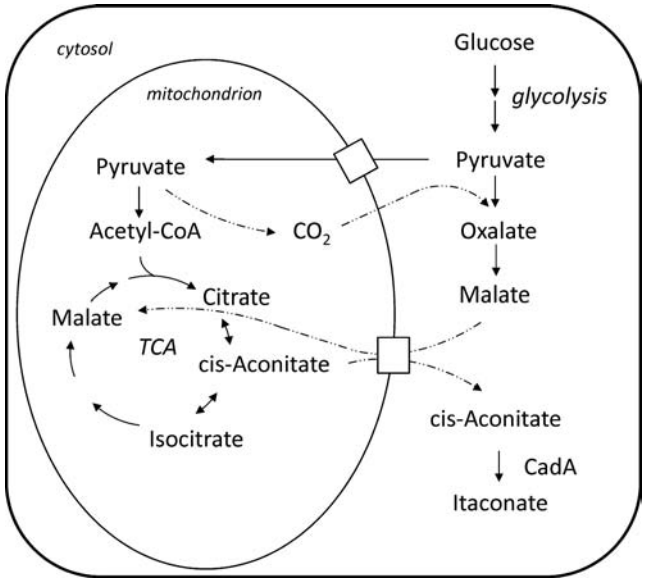

FIGURE 1 | Biosynthesis pathway of itaconate and its compartmentalization between cytosol and mitochondrion in the *A. terreus* cell; note: *cis*-aconitate transport is speculative.

of itaconic acid the compartmentalization of the pathway was analyzed by fractionized cell extracts distinguishing the enzymatic activity of a mitochondrial from a cytosolic enzyme. It was found that the key enzyme of the pathway, CadA, is not located in the mitochondria but in the cytosol (Jaklitsch et al., 1991), whereas the enzymes preceding in the pathway, namely citrate synthase and aconitase, are found in the mitochondria. However, a residual level of aconitase and citrate synthase activity is also found in the cytosolic fraction. The proposed mechanism is that *cis*-aconitate is transported via the malate–citrate antiporter into the cytosol (Jaklitsch et al., 1991). However, so far it was not shown whether *cis*-aconitate makes use of the mitochondrial malate–citrate antiporter or uses another mitochondrial carrier protein to be translocated to the cytosol.

Besides *A. terreus*, itaconic acid is known to be produced also by other fungi like *U. zeae* (Haskins et al., 1955), *U. maydis* (Haskins et al., 1955; Klement et al., 2012), *Candida* sp. (Tabuchi et al., 1981), and *Rhodotorula* sp. (Kawamura et al., 1981). No further investigations exist about the underlying reaction principles leading to itaconic acid formation in those species. However, recent evidence (Strelko et al., 2011; Voll et al., 2012) points into the direction that CadA activity constitutes the general pathway toward the formation of itaconic acid in nature. Very recently, itaconic acid was detected in mammalian cells, where it was found in macrophage-derived cells (Strelko et al., 2011). Those cells also possess a CadA activity and have the ability to form itaconic acid *de novo*. But, up to now no specific gene encoding this enzymatic activity was identified in mammalian cells.

However, the physiological role of itaconic acid in mammalian cells is still unknown. Strelko et al. (2011) speculate on the role of itaconic acid as an inhibitor of metabolic pathways, because it is described as an enzymatic inhibitor. On the one hand, itaconic acid is known to inhibit isocitrate lyase (Williams et al., 1971; McFadden and Purohit, 1977), which is the crucial part of the glyoxylate shunt, and thus can act as an antibacterial agent. On the other hand, itaconic acid can inhibit fructose-6-phosphate 2-kinase (Sakai et al., 2004) and thus have a direct influence on the central carbon metabolism. In rats it was shown that a itaconate diet leads to a reduced visceral fat accumulation, because of a suppressed glycolytic flux (Sakai et al., 2004).

ITACONIC ACID PATHWAY SPECIFIC ENZYMES AND GENES

The reaction catalyzed by the *cis*-aconitic acid decarboxylase was already described in 1957 (Bentley and Thiessen, 1957a,b). Subsequently performed ^{13}C and ^{14}C labeling experiments (Winskill, 1983; Bonnarme et al., 1995) confirmed the reaction scheme depicted in **Figure 2**. Itaconic acid is formed by an allylic rearrangement and decarboxylation from *cis*-aconitic acid removing either carbon C1 or C5 from the starting citric acid molecule (because of the symmetry of the molecule).

Furthermore, certain properties of the *A. terreus* CadA enzyme were determined: it has a K_m value of 2.45 mM (37°C, pH 6.2) and a pH optimum of 6.2 (Dwiarti et al., 2002). At pH 7.5 the activity drops significantly and is below 20% of the maximal value (Dwiarti et al., 2002). Until 2008, the sequence of the CadA protein was unknown, because the protein exhibits a general low stability. Kanamasa et al. (2008) were able to purify a substantial amount of the enzyme. By sequencing of the protein the N-terminal and four internal sequences were determined, which produced a single hit, ATEG_09971, in the genome database of *A. terreus*. The gene was named *cad1* and its protein product CadA. However, according to the nomenclature guidelines for *Aspergillus* it should rather be named *cadA* and CadA. The activity of the enzyme as a *cis*-aconitic acid decarboxylase (EC 4.1.1.6) was confirmed after heterologous expression of the gene in *Saccharomyces cerevisiae*. The CadA protein is a 490 amino acid protein (55 kDa) and has a high sequence identity with proteins from the MmgE/PrpD family, which includes 2-methylcitrate dehydratases. However, it is not clear, whether CadA has also a 2-methylcitrate dehydratase activity or whether a family member of the MmgE/PrpD class has also an activity as a *cis*-aconitic acid decarboxylase.

In contrast to the enzyme purification strategy, Li et al. (2011) used a transcriptomic approach to identify the *cadA* gene. A clone of the *A. terreus* strain NRRL1960 was cultivated at different conditions (pH, dissolved oxygen, etc.), which yielded different productivities and titers for itaconic acid. The conditions, which exhibited the highest difference in productivity and titer, were transcriptionally analyzed on a microarray with the assumption that genes involved in the itaconic acid pathway show an altered (higher) expression level during producing conditions. The *cadA* gene was highly scored in this analysis and thus can be identified in such an analysis. Interestingly another gene, encoding a mitochondrial carrier protein, was also highly scored in this analysis. This gene is located directly upstream of the *cadA* gene on the genome in *A. terreus*. Downstream of the *cadA* gene another transporter can be found which is annotated as a putative Major Facilitator Superfamily transporter. The mitochondrial carrier protein was detected in the transcriptomic analysis and was shown to have a direct positive influence on the itaconic acid production (Jore et al., 2011;

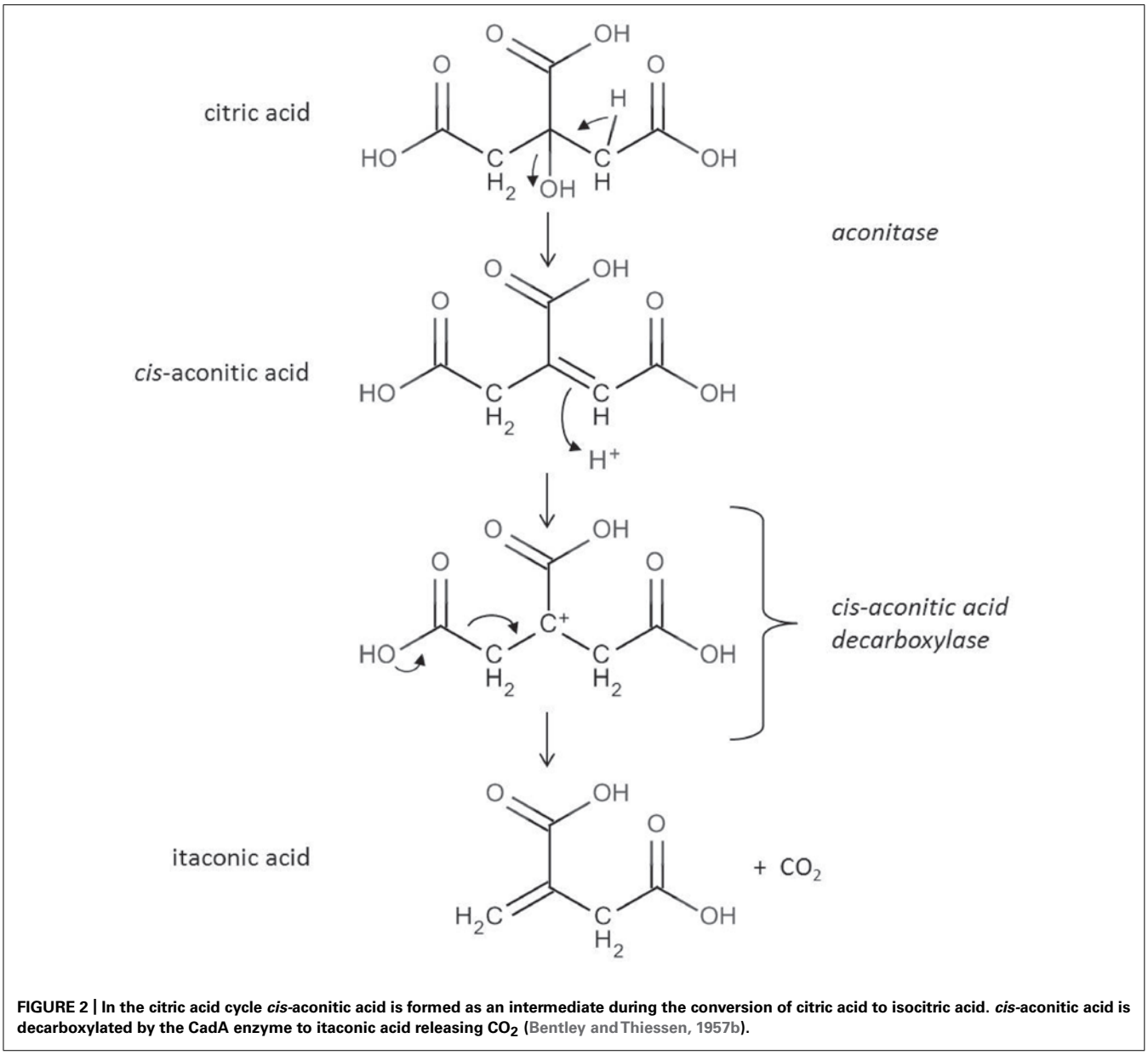

FIGURE 2 | In the citric acid cycle *cis*-aconitic acid is formed as an intermediate during the conversion of citric acid to isocitric acid. *cis*-aconitic acid is decarboxylated by the CadA enzyme to itaconic acid releasing CO_2 (Bentley and Thiessen, 1957b).

van der Straat et al., 2012). However, the mechanism and substrates of this putative transporter are still unknown and its role needs to be clarified, but it can be speculated that intermediates of the biosynthesis pathway like *cis*-aconitic acid are transported with this protein.

The activity of the *cis*-aconitic acid decarboxylase is crucial for the performance of the whole itaconic acid biosynthesis pathway. In an itaconic acid overproducing strain, which was obtained by an selection on high itaconate levels (Yahiro et al., 1995), five times higher transcription levels of the *cadA* gene were found than in a comparable wild type strain but no change in the amino acid sequence was detected (Kanamasa et al., 2008). Expressing the *cadA* gene in *A. niger* under various constitutive promoters of different expression strength demonstrated that the itaconic acid productivity directly correlates with the *cadA* transcript level (Blumhoff et al., 2013). It can be concluded that a high transcriptional level of this gene is essential for an optimal production performance. A high transcriptional level of the gene might be necessary, because of a low stability of the enzyme *in vivo*, which was found to be rather unstable *in vitro* (Dwiarti et al., 2002; Kanamasa et al., 2008).

CATABOLIZATION OF ITACONIC ACID

Much is known about the biosynthesis of itaconic acid and the underlying enzymatic mechanisms, but for a complete biochemical picture of a certain metabolite, also the knowledge about its degradation is necessary. Unfortunately, the information about the degradation pathway of itaconic acid is scarce. In mammalian cells (guinea pig and rat liver) it was found that itaconate is converted to itaconyl-CoA (Adler et al., 1957) and is further processed via

citramalyl-CoA (Wang et al., 1961) to pyruvate and acetyl-CoA. Hereby, it was found that malonate has an inhibitory effect and an addition prevents the degradation of itaconic acid (Adler et al., 1957). The first step of this degradation pathway can be catalyzed by the ubiquitous succinyl-CoA synthetase (Adler et al., 1957; Nagai, 1963; Schürmann et al., 2011). The third step of the pathway is catalyzed by a citramalyl-CoA lyase, where genes from *Chloroflexus aurantiacus* (Friedmann et al., 2007) and *Pseudomonas putida* (Jain, 1996) have been cloned. However, no protein and gene sequence was identified so far, which can catalyze the second step of the degradation pathway, which is an itaconyl-CoA hydratase (Cooper and Kornberg, 1964).

METABOLIC ENGINEERING OF THE ITACONIC ACID PATHWAY IN *A. terreus* AND *A. niger*

The levels of itaconic acid which were reached with *A. terreus* are currently limited to about 85 g/L. Although this is already a substantial amount it cannot be compared with the production of citric acid where titers over 200 g/L are steadily obtained in industrial processes. Transferred to the itaconic acid production a maximal theoretical titer of about 240 g/L should be achievable (Li et al., 2011). This goal could be reached by further breeding of currently existing strains or targeted genetic engineering.

In *A. terreus,* a gene was shown to influence the performance of itaconic acid production, which is a key enzyme of glycolysis. 6-phosphofructo-1-kinase is known to be inhibited by citrate and adenosine triphosphate (ATP). However, a truncated version of the *A. niger pfkA* gene was shown to exhibit a higher citric acid yield due to a reduced inhibition by citrate and ATP (Capuder et al., 2009). This truncated *pfkA* version had also a positive impact on the itaconic acid accumulation when expressed in *A. terreus* (Tevz et al., 2010). Another engineering approach deals with the intracellular oxygen supply. The production of itaconic acid requires continuous aeration and already a short interruption of oxygen decreases the itaconic acid yield. In order to reduce the sensitivity to oxygen a hemoglobin gene from *Vitreoscilla* was expressed in *A. terreus.* Indeed, the expression of this gene leads to an increased itaconic acid production. Furthermore, the strains exhibited a better recovery after the aeration was interrupted (Lin et al., 2004).

There is the possibility that the genetic make-up of *A. terreus* is not efficient enough to support the production of higher titers of organic acids. Therefore, a strategy is to genetically engineer the itaconic acid biosynthesis pathway into another host organism, which is already known to support the production of high titers of organic acids. As already mentioned, *A. niger* is such a candidate. The unique and crucial step in the biosynthesis pathway is the decarboxylation of *cis*-aconitic acid toward itaconic acid. When the *cadA* gene (Kanamasa et al., 2008) was characterized in *A. terreus* genetic engineering of the pathway into another organism became possible. Li et al. (2011) expressed the *A. terreus cadA* gene in *A. niger* strain AB 1.13. For this purpose, the *cadA* gene was placed under the control of the *A. niger gpdA* promoter, which enables a strong and constitutive expression. An *A. niger* strain which expresses the *cadA* gene alone has the ability to produce about 0.7 g/L itaconic acid. This level is not comparable with current production strains of *A. terreus*, but is a promising starting point for further engineering steps. Further attempts to rise the yield are to express genes like the above mentioned mitochondrial carrier protein together with the *cadA* gene (Jore et al., 2011; van der Straat et al., 2012).

OUTLOOK

Itaconic acid as a renewable organic acid is of growing interest for the chemical industry, because of its potential to replace crude oil based products like acrylic acid. Up to now, the microorganism based processes were improved by classical strain breeding and optimizations of the fermentation strategies and conditions. Especially the knowledge about the biotechnological process including oxygen supply, media compositions, and different bioreactor systems was significantly expanded (Kuenz et al., 2012). Regarding the media composition, it was found that copper ions positively influence the itaconic acid production in a genetically engineered *A. niger* strain (Li et al., 2012). However, it is not understood which biochemical reactions are responsible or involved in such an effect. As already mentioned above, the biochemical reactions and effects of itaconic acid in the production hosts are not fully described. The catabolization pathway of itaconic acid requires further investigations in order to engineer a production host with a disabled degradation pathway. The effect of itaconic acid on other metabolic pathways is also of interest because the understanding of its physiological role can prevent undesired side effects (toxicity, health risk, pathway inhibition) and increase the safety of its use. Furthermore, it can be an interesting target for medical research because in mammalian cells it was detected in a metastatic tumor cell line (Strelko et al., 2011). Further knowledge about its role as an enzyme inhibitor can help to develop less-resistant enzyme varieties like in the case of the phosphofructokinase 2. Another target for further engineering is the CadA enzyme, which is described as an unstable protein. Prolonging its *in vivo* stability can help to increase the efficiency of existing production hosts. Also the genetic regulation of the itaconic acid pathway in *A. terreus* requires a profound analysis. Li et al. (2011) have shown that genes involved in the biosynthesis pathway (*cadA*) can be identified by transcriptomic approaches. However, nothing is known so far about the regulatory mechanisms leading to the expression of those genes.

The investigations on the molecular principles of itaconic acid synthesis revealed that *cis*-aconitic acid decarboxylase is the dedicated step in its biosynthesis in *A. terreus.* Genetic engineering of this enzymatic step also renders other microbial hosts like *A. niger* to producers of itaconic acid.

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