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SYNTHETIC BIOLOGY TOOLS FOR METABOLIC ENGINEERING OF THE FILAMENTOUS FUNGUS PENICILLIUM CHRYSOGENUM

Fabiola Polli
ABSTRACT

Since the application of penicillin and other antibiotics, bacterial resistance to antibiotics developed hand in hand with their use in combating infectious disease. Therefore, there is an urgent need for novel molecules with unique structures to combat resistance towards existing antibiotics and that target new essential biological functions for antimicrobial therapies. With the recent developments towards an advanced synthetic biology toolbox for filamentous fungi, novel strategies can be applied for the discovery, production and modification of natural products into effective antibiotics.

INTRODUCTION

The discovery of penicillin by Alexander Fleming in 1928 generated a first understanding of the wide spread nature of the production of antibiotics and other bioactive compounds by filamentous fungi and encouraged research in this direction. While initial research was focused on natural product discovery and classical strain improvement (CSI), later on, it also became possible using recombinant DNA (rDNA) techniques to express heterologous genes in filamentous fungi for the production of semisynthetic antibiotics, such as cephalosporins 1.

One of the most important cell factories in antibiotics production is the filamentous fungus *Penicillium chrysogenum*. The initial isolate fungus *P. notatum*, did not produce enough of the antibiotic for mass production, and this initially slowed down the introduction of penicillins as anti-infectives. Therefore, classical strain improvement (CSI) through radiation and chemical mutation followed by selection, has led to strains that produced increased levels of β-lactams allowing the commercial application of this class of compounds and the exploitation of this fermentative process at industrial scale 2. The CSI resulted in many genomic alterations, such as: amplification of the penicillin biosynthetic gene cluster 3, increased amino acid metabolism 4, proliferation of microbodies that harbor the key enzymes involved in β-lactam synthesis 5, overexpression of various transporters and morphological changes that contribute to the efficiency of large scale fermentation 6. Interestingly, the CSI also resulted in the repression and inactivation of other secondary metabolites gene clusters 7, likely to divert nitrogen and carbon sources towards the increased production of the non-ribosomal peptide precursor of β-lactams, as well as to reduce pigment formation interfering in penicillin product recovery and purification. Recently, *Penicillium* species have been described that secrete a variety of secondary metabolites 8-9, but most have not been fully characterized or explored for possible pharmaceutical applications 10-11-12-13-14-15.

Additionally, a potentially interesting feature of the CSI improved *P. chrysogenum* strains is that they provide a great platform for the fermentative production of semi synthetic antibiotics, as exemplified by a metabolic engineering project on fermentative production of adipoyl-cephalosporins 16-17. This was realized by the introduction of a novel, heterologous enzyme, adipoyl-7-aminoacetoxyccephalosphoranic acid synthase and the feed of adipate as (β-lactam) side chain precursor, allowing the rapid development of a new generation of production strains of adipoyl-cephalosporins 18-19-20.
One drawback of the use of *P. chrysogenum* is the poorly developed genetic toolbox. In recent years, major advancements have been made to increase the efficiency of transformation and gene deletion, as well as of the use of plasmids to express heterologous genes. In this thesis, we will focus on the discovery of novel fungal compounds by deletion of two highly expressed groups of genes involved in secondary metabolites production and on the application of different synthetic biology techniques currently available for genetic engineering of filamentous ascomycetes, and in particular *P. chrysogenum*. We will also discuss how these techniques can be applied to further develop these organisms as cell factories for secondary metabolite production.

1. **FILAMENTOUS FUNGI**

Filamentous fungi are eukaryotic organisms and in the taxonomic group of Ascomycota, there is the extensive and important genera that includes *Aspergillus*, *Penicillium*, * Fusarium*, and *Claviceps* species. They can be found in soil, air, fruits and even in extreme environments such as the Antarctic ice core. Recently, new *Penicillium* species were found in marine environments, living in symbiosis with algae *Laurencia* and with sponges *Ircinia fasciculata* and *Chondrosia reniformis*. Filamentous Ascomycota are characterized by vegetative cells called hyphae and by sexual and asexual life cycles (Figure 1). The hyphal cells form compartments, which harbor various organelles, like nuclei, mitochondria and organelles with specialized functions like peroxisomes, glyoxysomes and woronin bodies.

These special organelles are also generically called microbodies and next to their structural function they play a role in several metabolic processes including primary carbon and nitrogen metabolism (e.g. fatty acids, methanol, alkanes, d-amino acids and purines), hyphal growth, spore germination and sexual spore formation. Microbodies are also involved in the production of secondary metabolites. In specific fungi like *P. chrysogenum* and *Aspergillus nidulans* the final steps of the β-lactam biosynthesis are catalyzed by microbody localized enzymes.

Ascomycota are characterized by the presence of a special structure called ascus where fusion of haploid nuclei and meiosis take place during sexual reproduction. However, an asexual cycle can also occur. Specifically, from a hyphal tip a single haploid spore called conidiospore is developed. Conidiospores are dispersed by the wind and under suitable conditions can germinate to form new vegetative hyphae. In *A. nidulans*, sexual reproduction results in the formation of a diploid ascus. In *P. chrysogenum*, the asexual cycle results in the formation of a conidium (or conidiospore), which can be dispersed by the wind and regenerate a new colony. The asexual cycle allows for rapid spread and adaptation to changing environmental conditions.

**Figure 1. Filamentous fungi A. nidulans life cycle.**

In the asexual cycle (orange arrows), from a vegetative mycelium (hypha), a spore called conidiospore (haploid nucleus) is released. In presence of favourable conditions the spores germinates and a new mycelium called homokaryon is formed. In the sexual cycle (pink arrows), in a ascogenous hypha, sexual spores (haploid nuclei) fuse together. After meiosis and post-meiotic mitosis haploid ascospores, eight in the case of *A. nidulans*, are formed and released from a structure called cleistothecium. Haploid nuclei carrying different genetics nuclei (black and white nuclei) can fuse together and an unstable heterokaryon is formed. By induction of special conditions in the parental nuclei, a balanced ratio of nuclei is ensured and the heterokaryon is maintained. If like genetics nuclei are combined a parasexual cycle (green arrows) occurs. The diploid homokaryon continue the cycle by mitosis followed by meiosis and the resulting haploid homokaryon can continue to develop in the sexual and asexual cycle.

*Figure from 30 with permission.*
conditions will germinate to form new mycelia. Furthermore, a so-called parasexual cycle can be present as observed for the first time in Aspergillus niger. Specifically, such cycle occurs when two homokaryons carrying haploid like nuclei fuse together and the resulting heterokaryon continues to divide first mitotically and then meiotically. A new haploid mycelium is formed and a new sexual or asexual cycle can begin again. Furthermore, if different nuclei of haploids fuse together, the resulting heterokaryon is unstable and a series of events, like haploidization and/or somatic crossing-over, occur to ensure the maintenance of the genome.

1.1. PENICILLIUM CHRYSOGENUM

Penicillium chrysogenum is a filamentous fungus that belongs to the genus Penicillium. In nature, it is a widely distributed mold often found on foods and in indoor environments. Under laboratory condition, the majority of this genus members reproduce asexually through chains of specialized hyphae, the brush-shaped conidiophores. Nevertheless, under very specific conditions, such as oatmeal agar supplemented with biotin, Penicillium species are able to sexually reproduce by induction of mating-type (MAT) loci. Penicillium chrysogenum forms a complex network of branched hypha, has green conidia, and sometimes secretes a yellow pigment. It can be used to produce secondary metabolites like roquefortine C, secalonic acids, meleagrin, chrysogine, PR-toxin, sorrentanone, xanthocillin X which is most famous for the production of several natural penicillin, β-lactam antibiotics, especially for commercial production of penicillins G and V.

The P. chrysogenum genome contains 13,653 ORF distributed over 32.19 Mb. The genome shows significant similarities with genomes of vegetative spores, called conidiospores, formed on the extension of specialized hyphae, the brush-shaped conidiophores. Nevertheless, under very specific conditions, such as oatmeal agar supplemented with biotin, Penicillium species are able to sexually reproduce by induction of mating-type (MAT) loci; 42, 43, 44.

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The P. chrysogenum genome contains 13,653 ORF distributed over 32.19 Mb. The genome shows significant similarities with genomes of other filamentous fungi. Of the 13,653 predicted proteins, approximately 60% could be attributed to functional protein classes as defined for genome sequences, e.g. related to metabolism, energy, cellular transport and other defined classes.

In recent years, P. chrysogenum has been renamed as P. rubens, but since the fungus is used for commercial purposes, the new name finds only slow acceptance in the field.

### 1.2. SECONDARY METABOLITES

While primary metabolites are essential and directly derived from central metabolism, secondary metabolites are not required for primary metabolic processes and growth of the cells. Production of secondary metabolites often occurs in a late phase of growth and is usually connected to sporulation, colony formation or other forms of cell differentiation. For example, in Alternaria alternata and Aspergillus nidulans, linoleic and melaminic acid derivatives are required for sporulation. During sporulation also toxic metabolites, such as mycotoxins are secreted. Furthermore, inhibition of sporulation has been associated with reduced aflatoxins production. In some Aspergillus species, the production of secondary metabolites is associated with the regulation of asexual and sexual spore development. Additionally, some secondary metabolites appear only after conidiation has been initiated. Examples of secondary metabolites and related functions are shown in Table 1.

**Table 1. Some functionally diverse fungal secondary metabolites**

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Fungal producer</th>
<th>Function</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyrothricin I</td>
<td>Aspergillus terreus</td>
<td>Sporulation induction</td>
<td>39</td>
</tr>
<tr>
<td>Cephalosporin</td>
<td>Cephalosporum acremonium</td>
<td>Antibacterial activity</td>
<td>40</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>Beauveria nivea</td>
<td>Immunosuppressor</td>
<td>41</td>
</tr>
<tr>
<td>Echinocandin</td>
<td>Aspergillus nidulans echinulatus</td>
<td>Anti-fungal</td>
<td>42</td>
</tr>
<tr>
<td>Ergotamine</td>
<td>Claviceps species</td>
<td>Vasoconstrictor</td>
<td>43</td>
</tr>
<tr>
<td>Fumagilin</td>
<td>Aspergillus fumigatus</td>
<td>Antifungal</td>
<td>44</td>
</tr>
<tr>
<td>Fusarin C</td>
<td>Fusarium moniliforme</td>
<td>Mutagen</td>
<td>45</td>
</tr>
<tr>
<td>Glucothricin</td>
<td>Aspergillus fumigatus</td>
<td>Genotoxicity</td>
<td>46</td>
</tr>
<tr>
<td>Intecrin acid</td>
<td>Xylosp.</td>
<td>HIV-1 integrase inhibitory activity</td>
<td>47</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>Aspergillus nidulans</td>
<td>Spore formation and development</td>
<td>48, 49</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>Aspergillus terreus</td>
<td>Cholesterol-lowering</td>
<td>50</td>
</tr>
<tr>
<td>Lysergic acid</td>
<td>Claviceps species</td>
<td>Hallucinogenic</td>
<td>51</td>
</tr>
<tr>
<td>Melanin analogs</td>
<td>Alternaria alternata, Cochliobolus heterostrophus, Aspergillus fumigatus</td>
<td>Spore survival and protection, Virulence</td>
<td>52, 53</td>
</tr>
<tr>
<td>Melagrin</td>
<td>Penicillium sp.</td>
<td>Antimicrobial activity</td>
<td>54</td>
</tr>
<tr>
<td>Mycotoxin</td>
<td>Aspergillus spp., Penicillium sp.</td>
<td>Mycotoxicosis activity</td>
<td>55, 56</td>
</tr>
<tr>
<td>Patulin</td>
<td>Penicillium urticae</td>
<td>Antimicrobial activity</td>
<td>57</td>
</tr>
<tr>
<td>Roquefortine C</td>
<td>Penicillium sp.</td>
<td>Antimicrobial activity</td>
<td>58, 59</td>
</tr>
<tr>
<td>Siderophore</td>
<td>Penicillium sp.</td>
<td>Affects fungal growth</td>
<td>60</td>
</tr>
<tr>
<td>Sorbicillinoid</td>
<td>Penicillium sp., Aspergillus porosulus, Tricoderma sp, Pheosporiopsis sp</td>
<td>Antimicrobial activity</td>
<td>61, 62</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>Fusarium gramineum</td>
<td>Sporulation induction</td>
<td>63</td>
</tr>
</tbody>
</table>
Historically, the systematic study of fungal metabolites started in 1922 with the identification of more than 200 compounds by Harold Raistrick. However, it was only after the discovery by Alexander Fleming of the first natural antibiotic Penicillin by the fungus *P. notatum* in 1928 that, extensive research on fungal metabolites began. Penicillin was widely used to cure wound infections during the second world war however, the early discovery of penicillin-resistant staphylococci strains followed by more antibiotic resistant strains led to a decrease of penicillin use and to a search for alternative antibacterial agents.

Further β-lactam compounds were discovered such as Cephalosporin C from a marine fungus, *Cephalosporium acremonium*. Between 1970 and 2010 many new bioactive compounds were isolated and characterized with antibiotic, antitumor, antifungal activity and found use as medicines, hormones or toxins, for human applications.

There is a large and complex diversity of secondary metabolites and associated biosynthetic pathways. A method for classification is based on carbon and nitrogen sources, together with precursors derived from primary metabolism such as acetyl-CoA and amino acids that are utilized in the secondary metabolites pathways.

Essentially, secondary metabolites can be classified in three main groups: 1. Amino acid derivatives and non-ribosomal peptides (NRP); 2. Fatty acids derived compounds and polyketides; 3. Terpenes and Indole alkaloids. In the first group, proteogenic and non-proteogenic amino acids are utilized by large multi domain nonribosomal peptide synthetase (NRPS) enzymes to synthesized non-ribosomal peptides (NRP). Ribosomes are not involved in the formation of NRP, and these NRPs can have different lengths, be linear or cyclic, and further be modified by accessory enzymes. Examples are peptaibols and cyclosporine as well as a variety of mycotoxins called roquefortines, and analogues like melagrin and glandicolin (Figure 2).

In the second group, acetyl-CoA and malonyl-CoA are utilized by polyketide synthetase (PKS) enzymes to synthesize corresponding polyketides. A few examples are the hypolipidemic agent compactin and lovastatin or the pigments fusarubin and bikaverin. The mevalonate pathway provides isoprene units that are used by terpene cyclases to form terpenoids, the third class of secondary metabolites. They can be linear or cyclic, such as carotenoids and gibberellins. The aromatic amino acid tryptophan and dimethylallyl pyrophosphate are used to produce indole alkaloids and related compounds, that are mainly produced by the fungus *Penicillium* and the parasitic *Claviceps*.

In nature, secondary metabolites may be mediators for communication, growth inhibitors and habitat protectors. Indeed, fungi live in complex ecosystems where they interact with other fungi and organisms, such as bacteria, algae, protozoans and metazoans and plants. Secretion of secondary metabolites with toxic properties provide a potential competitive advance over other organisms.

Genes responsible for the production of secondary metabolites are often clustered. Most likely, filamentous fungi obtained such gene clusters or parts thereof from bacterial sources through horizontal transfer. Mutation and natural selection contributed to the diversification of the compounds produced. Examples of mutations that induced diversity were found in the terpene gene cluster and in genes that code for the multi modular NRPS and PKS enzymes.
1.3. SECONDARY METABOLITES GENE CLUSTERS

Mostly secondary metabolite gene clusters encompass 10-25 KB and are co-located on a single chromosome, although there are exceptions. For the biosynthesis of meroterpenoids by *A. nidulans* two separate gene clusters are needed that are located on different chromosomes. Importantly, there is an enormous diversity of biosynthetic gene clusters and these are mostly not common in all fungi.

Fungal secondary metabolites gene clusters are readily recognized by the presence of genes encoding for the key enzyme, which is either a NRPS, PKS, terpene cyclase, or prenyltransferase. These are individual classes of enzymes, but each consists of a conserved architecture that can be easily recognized by bioinformatics means. Adjacent genes are responsible for the further tailoring of the primary product, and this may include reactions like oxidation, reduction and methylation. Additional genes are needed for regulation and the secretion of the secondary metabolites.

Multi modular enzymes consist of domains that are responsible for various sub reactions that work in concert. For example, a minimal NRPS module consists of an adenylation (A) domain responsible for amino acid activation, and a thiolation (T) domain also known as peptidyl carrier protein (PCP) that serves as an anchor for the growing peptide chain and a condensation (C) domain that is responsible for transfer and peptide growth. There can be further domains, such as a thioesterase (TE), methyltransferase (MT) or epimerisation (E) domain. Similarly, PKS enzymes contain a ketoacyl synthase (KS) domain for decarboxylation of the extender unit, an acyl carrier protein (ACP) for extender unit loading and an acyltransferase (AT) domain for extender unit selection and transfer. Additionally, domains encoding enoyl reductase (ER), β-ketoacyl reductase (KR), methyltransferase (MT), thioesterase (TE) and dehydratase (DH) activity can be present to further process the polyketide synthesized. PKS and NRPS units (modules) can cooperate in so-called hybrid NRPS-PKS enzymes. A few examples of hybrid NRPS-PKS enzyme products are rapamycin, yersiniabactin, myxovirescin (also known as antibiotic TA), the cyclic pentapeptide myxochromides S1–3 and the antitumor agent epothilone.

The availability of fungal genome sequences in combination with modern gene prediction software like SMURF (www.jcvi.org/smurf/) and AntiSMASH (http://antismash.secondarymetabolites.org/) have led to a quick identification of numerous genes and gene clusters, putatively responsible for secondary metabolite production. Recently, a systematic deposition and retrieval system on data on biosynthetic gene clusters has been established, i.e., the minimum information about a Biosynthetic Gene cluster (MIBiG) data standard. This will prevent redundancy and serves as a quick resource to determine if compounds have been described before. Interestingly, a large proportion of the identified gene clusters are not expressed under typical laboratory culture conditions. For example, the genome of *P. chrysogenum* encodes ten NRPS, twenty PKS and two hybrid NRPS-PKS genes, but only four NRPS genes are expressed under standard laboratory conditions, the Pc21g21390 (pcbAB), Pc21g15480 (roqA), Pc21g12630 (chryA) respectively from penicillin, roquefortine, chrysogine gene cluster and Pc16g04690 (hcpA) that encodes a fungisporin. Three out of these four genes are involved in the production of the mycotoxin roquefortine, the cyclic tetrapeptide fungisporin, and the yellow pigment chrysogin, which inspired the scientific name of this fungus. Therefore, classical methods to identify new metabolites and their corresponding biosynthetic genes, such as gene inactivation and comparative metabolic profile analysis are not suitable for the so-called cryptic or silent gene clusters. Nevertheless, the development of new genetic tools now offers novel solutions for the discovery, optimization and production of bioactive molecules as it will be discussed in the next section.

2. GENETIC TOOLS TO STUDY FILAMENTOUS FUNGI

Filamentous fungi have a large impact on human life since they have been widely used for the industrial production of diverse enzymes or metabolites. However, due to typical filamentous fungal features, such as the multicellular and multinuclear mycelium morphology, and because of the lack of sufficient suitable selection marker and plasmids, genetic engineering approaches for filamentous fungi are less efficient compared to...
those available for bacteria and yeast \textsuperscript{145}. Nevertheless, the availability of complete genome sequences \textsuperscript{5, 146, 147}, and the deletion of proteins involved in non-homologous end joining (NHEJ) pathway have vastly contributed to improve the precise design and generation of genetically modified strains. The generation of new experimental transformation strategies for the specific and unspecific integration of DNA sequences into the genome and the development of several novel CRISPR/Cas genome editing methods have allowed the specific manipulation of gene expression and function in a variety of filamentous fungi. Furthermore, new synthetic biology tools have been exploited for modular assembly of genes and pathways, such as novel promoters and terminators libraries as well as autonomously, stable replicating plasmids, which can be used as a vector for synthetic pathway reconstruction.

The genetic tools currently available for metabolic engineering of Penicillium strains are discussed in the following sections.

### 2.1. METHODS FOR GENETIC TRANSFORMATION

With the understanding that filamentous fungi are a useful resource for novel bioactive compounds such as the penicillins, further research focused on understanding of physiological and genetic aspects of industrially important fungi. Initially, this meant the development of methods for highly efficient genetic transformation to facilitate uptake of exogenous DNA and to have more control to direct metabolism and other features of these fungi. In 1973, the first transformation of Neurospora crassa was reported \textsuperscript{149}, which a decade later was followed by transformation methods for \textit{A. nidulans} \textsuperscript{149, 150, 151}. DNA uptake was achieved mainly by using protoplast and Polyethylene glycol/CaCl\textsubscript{2}. Protoplasts are readily obtained from fungal mycelium by means of enzyme cocktails containing various cell-wall degrading enzymes \textsuperscript{36}. To increase the DNA delivery, protoplasts were also fused to liposomes, synthetic lipid vesicles that have been shown to enhance transformation efficiency \textsuperscript{153}. However, not all filamentous fungi show efficient formation and regeneration rates of protoplast \textsuperscript{164}. Therefore, new types of transformation protocols were introduced utilizing lithium acetate (LiAc) treatment \textsuperscript{148}, electroporation \textsuperscript{155} or a biolistic particle delivery system methods (gene gun) \textsuperscript{156, 157}.

LiAc treatment does not depend on protoplast formation, but on cell permeability induced by Li\textsuperscript{+} ions thereby, increasing the transformation efficiency \textsuperscript{158}. Electroporation is based on the application of high voltage on protoplasts and conidia resulting in 50% of cell death, which influence the transformation efficiency \textsuperscript{159}. The gene gun method exhibits increased targeted delivery and genetic stability, due to the direct delivery of DNA in the cell by using super-speed tungsten or gold particles. However, the tedious optimization of numerous factors negatively influence the transformation efficiency \textsuperscript{160}.

### 2.2. SELECTION MARKERS

An important requirement for efficient transformation and transformants selection are specific marker genes. A series of marker genes are available for filamentous fungi. For instance, marker genes \textit{niaD} (encodes nitrate reductase) and \textit{pyrG} (encodes orotidine-5-phosphate decarboxylase) have been widely used in transformation of \textit{Aspergillus} species \textsuperscript{154, 164, 165}. However, to use these markers, host strains are required that either have inactive gene variants or lack these genes to allow for selection on nitrate or uracil respectively. Acetamidase encoded by the \textit{amdS} gene of \textit{A. nidulans} allows fungi to use acetamide as sole nitrogen source. This gene was used for the first time in transformation of \textit{A. nidulans} \textsuperscript{164} and \textit{A. niger} \textsuperscript{165}. Fungi transformed with the \textit{amdS} and \textit{pyrG} genes are sensitive to fluoroacetamide and 5-fluoroorotic acid (5-FOA), respectively. Thus, these selection markers can be eliminated by counter selection and the resultant strain can then be used for further transformation. On the other hand, marker selection and counter selection are not straightforward procedures, as often many rounds of sporulation are needed followed by growth on selective medium for strain purification, because of the multi nuclei features of filamentous fungi \textsuperscript{166}. Further selection markers are based on bacterial antibiotic resistance genes such as \textit{ble} (phleomycin), \textit{hph} (hydromycin) and \textit{nat} (nourseothricin) that are placed under control of a fungal promoter \textsuperscript{167, 168, 169}. Such dominant selection markers can readily be used but also spontaneous resistance to the drugs may occur, while proper growth conditions need to be used to prevent significant background growth. Therefore, fungal transformation and selection can be laborious, time consuming and with low reproducibility.

The restricted number of selectable marker genes that are available for filamentous fungi hampers multiple gene modifications. This drawback has been addressed using site-directed recombination technology tools such as the yeast FLP/FRT \textsuperscript{170}, \textit{β-rec/six} \textsuperscript{171} and the bacteriophage Cre-loxP recombination system \textsuperscript{172} or the CRISPR/Cas9 system \textsuperscript{173, 174} (See section 2.6).
2.3. CHROMOSOMAL DNA INTEGRATION

To introduce DNA into the fungal genome, homologous or heterologous recombination events are used. Homologous integration may occur via a single or double cross-over event, using DNA fragments carrying the upstream and downstream flanking sequences of the target gene and the selection marker gene. In this way, it is possible to realize gene disruption or deletion events. Often, this process occurs with a very low frequency, compared to heterologous integration, and typically long flanking regions are required (>1000 kbase). To increase the efficiency of homologous integration, Agrobacterium tumefaciens-mediated transformation (AMT) was developed. Specifically, this Gram negative soil bacterium contains Ku70, Ku80 protein dimers are involved in the direct ligation of the double stranded break ends of DNA.

Consequently, the need to understand the molecular mechanism of the ATM transformation led to the characterization of the non-homologous end-joining (NHEJ) DNA-repair pathway. A multi-subunit complex, where Ku70, Ku80 protein dimers are involved in the direct ligation of the double stranded break ends of DNA.

In a NHEJ deficient strain the efficiency of homologous recombination is markedly increased, up to 100% while shorter homologous flanking regions (-500 bps) can be used.

Heterologous recombination in filamentous fungi is relatively efficient and leads to mitotic stability. However, this method has the risks that multiple copies of a specific gene are incorporated while expression is not only affected by the copy number but also influenced by the integration position. Multi copy transformants can be obtained by co-transformation of non-selected plasmids carrying the target sequence and a plasmid that harbors the selection marker. This can result in higher levels of expression and mitotic stability. The disadvantage is that the copy number cannot be controlled and chromosomal integration occurs at undefined sites.

2.4. SPECIFIC CHROMOSOME EDITING METHODS

To study gene and protein functions and interactions, a set of molecular tools and strategies are available. For example, mutagenesis is an efficient method to investigate gene function and to observe related phenotypic changes in fungi. Considerable mutant frequencies may be achieved by increasing the target sites for recombination by means of a restriction enzyme mediated integration (REMI) mutagenesis strategy. In the REMI procedure, mutations can be generated by random insertions of DNA fragments into the fungal genome that has been treated with the same restriction enzymes used to generate the exogenous fragments. This technique, which relies on protoplast transformation, was applied for the first time with S. cerevisiae and later used widely for the simultaneous mutagenesis and tagging of genes in filamentous Ascomycete such as Cochliobolus heterostrophus.

A functional equivalent of the REMI procedure is Transposon arrayed gene knockout (TAGKO). The TAGKO technique is based on the use of homologous or engineered heterologous transposons (TE) which are ubiquitous mobile genetic elements that can be easily transferred into heterologous hosts and therefore, do not require a high frequency fungal transformation approach.

Specific and targeted DNA modification tools are very useful for the precise editing of the genome. For this purpose, enzymes derived from bacteria and fungi that are able to induce site-specific recombination events can be used. Recombinases, such as CRE/FLP, are able to specifically recognize short nucleotide target sequences. With LoxP/FRT this concerns an asymmetric 8 bp spacer flanked by 13 bp inverted repeats and when two of such structures are present, specific recombination events can be induced (Figure 3).

These techniques were successfully used for genetic engineering in yeast, mammals and filamentous fungi, since they can be used to introduce insertions, deletions, inversions and translocations at specific sites in the genome.

Another strategy to induce specific targeting and modification of defined DNA sequences in vivo is by site-specific nucleases. Specifically, these engineered nucleases induced double-strand breaks at a target site location in the genome that is then successively repaired by the nonhomologous end-joining (NHEJ) or homologous recombination (HR) systems resulting in a specific mutation.

There are four classes of engineered nucleases that are frequently used
Genetic tools to study filamentous fungi

Synthetic biology tools for metabolic engineering of the filamentous fungus Penicillium chrysogenum

DNA assembly

for this purpose: Meganucleases, Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector-based Nucleases (TALEN), and the CRISPR/Cas system. Meganucleases recognize a stringent DNA sequence (>14bp) thus, they cause less toxicity in cells compared to non-specific nucleases. However, the number of specific meganucleases is limited and their construction to cover all possible sequences is a costly and time-consuming activity. Therefore, alternative approaches using zinc finger nucleases (ZFNs) and engineered meganuclease were developed. These methods are based on the recognition of specific nucleotides by a complex of a zinc finger protein and a nonspecific DNA-cleaving enzyme fused to a FokI sequence-specific recognition endonuclease.

Lately, an alternative and readily programmable DNA binding domain was used, the Transcription Activator-like Effector Nucleases (TALENs).

The TAL effector proteins, provides a DNA-binding domain with less stringent binding requirements compare to ZFNs but they may also causing off-target mutations. Moreover, ZFNs and TALEN-based approaches can be used to modify defective genes in the genome, which is a so-called gene therapy practice. Examples of in vivo and in vitro gene corrections are the repair of the interleukin-2 receptor common gamma chain (IL-2Rγ) and the X-linked severe combined immunodeficiency (X-SCID) in mammals.

Although the aforementioned studies with nucleases provide very efficient genome editing techniques, a main breakthrough was achieved by a RNA guided double-strand break induction system named CRISPR/Cas9 system. This bacterial based system relies on DNA recognition mediated by a single guide RNA (sgRNA) and on nuclease Cas9 that is directed to the target DNA sequence by the sgRNA. In filamentous fungi, the CRISPR/Cas9 system can be carried by an AMA fungal vector and it can lead to nonspecific mutations or to specific gene integration at the genomic locus of interest by the non-homologous end-joining (NHEJ) or by the homologous recombination (HR) systems, respectively. In the latter case, donor DNA is co-transformed with the AMA fungal vector.

In fungi, only few RNA polymerase III promoters responsible for sgRNA expression have been identified. Therefore, the sgRNA is expressed as a chimeric larger RNA transcript by RNA polymerase II and then later converted by ribonuclease cutting on sites engineered in the sgRNA expression construct. Moreover, there are several types of Cas nucleases that have been used and probably many more types remain to be discovered. Since they can cleave nearly any DNA sequence complementary to the guide RNA, they make gene editing very simple. Therefore, the CRISPR/Cas system was successfully applied in numerous organisms as diverse as humans, plants, parasites and microbes, including several filamentous fungi.

2.5. DNA ASSEMBLY

To build genomic libraries or biosynthetic gene clusters, often several smaller DNA elements such as promoters, ORFs and terminator sequences, have to be carefully assembled together into larger functional gene or biosynthetic gene cluster expression units. This laborious process requires highly efficient, simple and cost-effective assembly strategies.
Genetic tools to study filamentous fungi

In recent years several methods have been developed to rapidly assemble two or three fragments into a linear gene expression cassette. A common and widely used method is based on restriction enzyme-based assembly. Because of its simplicity, this method is used for more complex assemblies and scar-less systems such as Golden Gate, BioBrick™, and BglBricks. The implemented assembly systems show good performance with multiple fragments, but a major drawback is still incomplete digestion/ligation. Nevertheless, these methods have been used for a wide range of applications from plasmid library construction to synthetic metabolic pathway assembly in several organisms, including filamentous fungi.

The system that ensures the best performance with multiple (>25) long (up to several hundred bps) fragments is based on the use of recombinase or exonuclease in vivo. The first in vivo recombination system was used in E. coli to construct a bacterial artificial chromosome (BAC) vector followed by more efficient recombination systems in yeast that were used to construct a yeast artificial chromosome (YAC) vector. Only very short homologous sequences (>25 bps) are needed for homologous recombination in S. cerevisiae and this can be easily achieved by PCR. In recent years, efficient and accurate in vitro recombinase-based technologies have led to new cloning systems such as In-Fusion, Gateway™, and BioCat™ Cold-Fusion. In addition, fusion PCR or overlap extension PCR (OE-PCR) methods were developed.

In recent years even simpler and highly effective cloning methods were developed such as PIPE (polymerase incomplete primer extension), SHA (successive hybridization assembly) and OSCAR (one-step construction of Agrobacterium-recombination-ready plasmids). These PCR methods are successful in building gene targeting cassettes with large homologous flanking regions (>1 Kb), that can be used for the transformation of filamentous fungi.

Another in vitro assembly method based on a PCR reaction is the Gibson isothermal assembly. In this method DNA fragments carrying 20-40 bps overlaps are mixed with exonuclease, DNA polymerase, and DNA ligase and incubated at 50°C for up to one hour, resulting in a unique DNA assembly fragment. This powerful cloning method shows a high efficiency when it concerns large (>20 Kb) assemblies, but it is costly and recombinase dependent. However, the combination of Gibson cloning with in vivo recombination in YACs allowed the creation of the first artificial Mycoplasma genitalium genome.

Successful applications of the aforementioned modular assembly tools concern the multi modular polyketides (PKS) and non-ribosomal (NRPS) enzymes. In fact, to expand the molecular diversity of the pharmacologically important produced metabolites, a variety of modular combination and modifications have been investigated employing modular assembly techniques.
2.6. PROMOTORS AND TERMINATORS

Important elements of a synthetic biology toolbox are promoters and terminators that vary in strength. Many studies aim to improve the level of protein expression in microbes. Typically, both endogenous and exogenous promoters have been used for this purpose. Specifically, during the past two decades, gene (open-reading-frame, orfs) sequences from higher eukaryotes, such as mammals and plants and even bacteria, have been expressed in Aspergillus and in Trichoderma. For instance, the mammalian chymosin gene has been expressed in A. nidulans, using the A. niger glucoseamylase promoter (glaA) and lysozyme and glucoseamylase were expressed in A. niger employing the A. nidulans glyceraldehyde-3-phosphate dehydrogenase promoter (gpdA). Recently, a set of promoters was tested and characterized on, inducibility, timing and level of expression, using a reporter system that can be used in P. chrysogenum.

However, often strong expression is not recommended, especially for the production of bioactive natural products like antibiotics and toxins. Then, the expression of a gene needs to be tightly tuned. One of the promoters of the alcohol region is the alcohol dehydrogenase alcA which is easily regulated by the presence of alcohols/ketones and lactose/glycerol that induce or repress, product formation respectively. This system was used successfully to express endoglucanase and interferon α2 in A. nidulans. Another example of a tunable expression system that uses metabolism-independent promoters is the Tet-on/off. The system was applied for several model fungi like A. niger and A. fumigatus. The tetracycline transactivator (tTA) or the reverse tetracycline transactivator (rtTA2s-M2) are controlled by metabolism-independent promoters like xyl (xylose) or gpdA (glyceraldehyde-3-phosphate dehydrogenase) and are able to bind to DNA at specific TetO operator sequences that are usually upstream the promoter of interest. The presence or absence of the antibiotic tetracycline or one of its derivatives (e.g. doxycycline) regulates the binding of tTA and rtTA2s-M2 to the TetO sequences. This artificial gene expression system can be envisioned for many applications such as gene therapy and for controlled protein production in microbial production strains.

Besides promoters, terminator sequences are other important features in the construction of gene expression systems for homologous and heterologous proteins expression. Therefore, terminator sequences from several filamentous fungi have been investigated and used in the construction of expressing cassettes. Selected examples are the A. nidulans trpC, the N. crassa arg-2 and the A. nidulans AN4594.2 and AN7354.2.

2.7. AUTONOMOUSLY REPLICATING PLASMIDS

Many bacteria and yeast species harbour natural plasmids that carry autonomously replicating sequences (ARSs) allowing the replication of the plasmid. These plasmids can be used with an appropriate selection marker to introduce new genes into the host cell with high frequency. In contrast, in filamentous fungi, plasmids are almost completely absent. A. nidulans possess an ARSs termed AMA sequence that confers autonomous replication of plasmid vectors in several filamentous fungal species. However, this type of plasmid was used so far only to a limited extent, because of poor stability and the risk that the plasmid integrates into the genome. Furthermore, to obtain more stable plasmids, centromeric and telomeric sequences were investigated in filamentous fungi. Linear plasmids containing telomeric sequences were constructed and have been shown to increase the transformation efficiency of the filamentous ascomycete Fusarium oxysporum and Nectria haematococca several thousand fold. However, the autonomous plasmids were unstable without selection and they were poorly transferred during cell division. One potential application of these telomeric sequences was to combine them with centromere sequences (and, or AMA sequences) to construct artificial fungal chromosome vectors, like the well-known yeast artificial chromosomes (YAC) vectors. Such vectors would allow the use of large fragments of DNA in the construction of genomic libraries for biosynthetic gene clusters and redesigned metabolic pathways. However, not many fungal centromere sequences have been identified and characterized so far.

Generally, plasmid can be maintained by the use of a selective marker, which has the downside to be costly due to the continuous use of antibiotic into the medium. Additionally, only a series of marker genes are available for filamentous fungi. The design of novel type of mitotic stable plasmids that are marker independent represent one of the future tools in fungal synthetic biology research.

CONCLUDING REMARKS

The battle against multidrug resistant bacteria provokes an urgent need for novel antibiotics based on novel, unique core structures. Filamentous fungi fulfill an important role in industrial biotechnology because of their use for the production of a broad range of enzymes and natural products.

Autonomously replicating plasmids
products. For about two decades, molecular genetic tools have enabled us to engineer these organisms for production metabolites and enzymes by expressing extra copies of both endogenous and exogenous gene. However, despite their importance only few model fungi have been studied in detailed and only relatively few genetic tools are currently available.

For that reason, a challenge for the future is to develop and use more advanced synthetic biology tools for a broader range of fungi. In fact, these tools can be applied to engineer novel filamentous fungal strains for the expression of newly designed biosynthetic pathway, to discover, modify and characterize novel natural products, hopefully including novel structures with antimicrobial-antibiotic activities.

**SCOPE OF THIS THESIS**

Classical strain improvement (CSI) has had a big impact on the development of *Penicillium chrysogenum* as an industrial strain. This involved mostly random mutagenesis and selection. However, as of now new synthetic biology methods have hardly been applied to enhance the industrial potential of this fungus. In this thesis, we aim to expand the set of genetic tools for metabolic engineering of the filamentous fungus *Penicillium chrysogenum*. Furthermore, we describe the design of an efficient host strain that can be used for the identification of novel secondary metabolites and for the production of natural and unnatural compounds.

**Chapter 1** describes an introduction to filamentous fungi with the specific emphasis on *P. chrysogenum*, and gives some insights on the metabolites produced by the secondary metabolism of filamentous fungi and the genetics behind these metabolites production. This chapter also describes the genetic toolbox available for engineering ranging from DNA assembling and editing methods to promoter parts and autonomously replicating plasmids.

**Chapter 2** describes a method for the generation of a secondary metabolite free strain by deletion of two highly express secondary metabolites gene clusters, chrysogine and roquefortine in a strain of *P. chrysogenum* that was already cleared from its multiple penicillin gene clusters. The engineered strain shows that the deletion of the chrysogine gene cluster resulted in increased levels of roquefortine metabolite production without affecting the expression of the core NRPS enzyme of the roquefortine gene cluster. Moreover, the secondary metabolite deficient strain produces novel metabolites that have not yet been associated with a specific secondary metabolite gene cluster.

**Chapter 3** presents an inventory of possible promoters and their strength for use in *P. chrysogenum*. This inventory is based on a modular reporter system employing the red fluorophore DsRed under control of a specific *Aspergillus* and *Penicillium* promoter, which acts as an internal standard and the green fluorescent protein gene under control of one of the selected promoters. These vectors were constructed as synthetic pathways using Golden gate and in vivo homologous recombination in the yeast *Saccharomyces cerevisiae*, and transferred into *P. chrysogenum*. Subsequent strains were analyzed in the Biolector system, which provides a semi high throughput fermentation system that allows on-line monitoring of various parameters. The inventory of promoter strengths adds to the synthetic toolbox development.

**Chapter 4** describes the refactoring of the penicillin biosynthetic gene cluster in a *P. chrysogenum* strain lacking this cluster. In addition, the chapter describes an AMA plasmid based expression system that is stably maintained in cells due to the presence of an essential gene. This plasmid acts as a novel platform for metabolic engineering approaches. Herein, the β-lactam pathway which comprises three genes (*pcbAB, pcbC and penDE*) was reassembled from large DNA fragments using in vivo recombination in *P. chrysogenum*. The pathway was targeted into original pen locus, an alternative chromosomal location and the AMA vector, and penicillin production levels were compared. The pathway refactoring is a first step toward the modification of the penicillin biosynthetic gene cluster for the production of alternative β-lactam antibiotics.

**Chapter 5** provides a summary and presents future perspectives of the work described in the thesis.