Synthetic and Systems Biotechnology 1 (2016) 143–149

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology/

Study on genetic engineering of *Acremonium chrysogenum*, the cephalosporin C producer

Youjia Hu*, Baoquan Zhu

China State Institute of Pharmaceutical Industry, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 13 April 2016
Received in revised form
11 August 2016
Accepted 11 September 2016

Keywords:
Acremonium chrysogenum
Cephalosporin C
Genetic engineering
Molecular breeding

ABSTRACT

Acremonium chrysogenum is an important filamentous fungus which produces cephalosporin C in industry. This review summarized the study on genetic engineering of *Acremonium chrysogenum*, including biosynthesis and regulation for fermentation of cephalosporin C, molecular techniques, molecular breeding and transcriptomics of *Acremonium chrysogenum*. We believe with all the techniques available and full genomic sequence, the industrial strain of *Acremonium chrysogenum* can be genetically modified to better serve the pharmaceutical industry.

Contents

1. Introduction

Acremonium chrysogenum, belongs to Filamentous fungi, is an important industrial microorganism. One of its metabolites, cephalosporin C (CPC), is the major resource for production of 7-amino cephalosporanic acid (7-ACA), an important intermediate for manufacturing of many first-line anti-infectious cephalosporins-antibiotics, in industry.

Cephalosporins belong to the family of beta-lactam antibiotics. Compared with the first-discovered penicillin, cephalosporins have obvious advantages since they are more resistant to penicillinase and are more effective to many penicillin-resistant strains. The incidence of adverse effects for cephalosporins is also lower than that for penicillins and other anti-infectious agents. Thus, cephalosporins are among the most-widely used anti-infectious drugs in clinic.

In China, the research & development of cephalosporins started from 1960s, and cefoxitin was first developed in 1970. In the past 40 years, cephalosporins-antibiotic is one of the most developed medicines in Chinese market. It counts for more than 40% of the anti-infectious market share.

As the major resource for manufacturing 7-ACA, the production and cost of CPC is of the utmost importance for the cephalosporins-antibiotic market. The Ministry of Science and Technology of China has listed the fermentation of CPC as the key scientific and technical projects during the past 30 years due to the continuous demanding

* Corresponding author.
E-mail address: huyoujia@sinopharm.com (Y. Hu).

http://dx.doi.org/10.1016/j.synbio.2016.09.002

of strain improvement for CPC-producing *Acremonium chrysogenum*.

Because of the limitation of traditional techniques on strain improvement for *A. chrysogenum*, along with the ubiquitous applications of molecular biology, genetic engineering has become a powerful tool to manipulate the antibiotic producing strain and to obtain high-yield mutant strain.

This year, we will celebrate the 90th birthday of Dr. Arnold Demain, the leader of β-lactam antibiotics research and development [1]. This review will focus on the study of genetic engineering of *A. chrysogenum* in our lab and other groups to memorize the achievements of Dr. Demain's contribution in this field.

2. Biosynthesis of CPC

The industrialization of CPC fermentation has been established years ago with the breakthrough in key technologies including fermentation yield, fermentation regulation and preparation & purification. Dr. Arnold Demain is truly a pioneer and a founder in discovering the biosynthesis of CPC [2,3] such as stimulation by methionine [4] and its precursors [5]. Nevertheless, there are still a lot of recent publications on improvement of CPC-producing strain by traditional methods, such as UV [6] or NTG [7] mutagenesis, and optimization of fermentation process [8]. More recently, glycerol was found can be used in the fermentation broth [9,10] replacing methionine that Dr. Arnold Demain used decades ago, to stimulate the biosynthesis of CPC. Researchers also tried solid-state and submerged fermentation of CPC [11] besides the traditional production. However, most of the latest strain breeding techniques are at molecular level. And the most important approach is the research on biosynthesis of the target metabolite.

Demain's group has studied on the role of methionine in the fermentation of CPC [12]. Methionine can stimulation the production of CPC in the crude media, and the addition of methionine was still kept in current *A. chrysogenum* fermentation. They found that methionine does not repress or inhibit cysteine metabolism. Although methionine can upregulate the transcriptions of *pcbAB*, *pcbC* and *cefEF*, and stimulates the mycelial fragmentation, the mechanism behind the fact still needs to be investigated [13,14].

The biosynthesis of CPC during the fermentation of *A. chrysogenum* has been well elucidated now. There are two gene clusters on the chromosome that involved in the biosynthesis of CPC. The “early” cluster consists of *pcbAB-pcbC* and *cefD1-cefD2*. *pcbAB-pcbC* encode two enzymes responsible for the first two steps in CPC biosynthesis [15]. *cefD1-cefD2* encode proteins that epimerize IPN to penicillin N [16]. The “late” cluster consists of *cefEF* and *cefG*, which encode enzymes responsible for the last two steps [17].

The biosynthesis pathway of CPC is illustrated in Fig. 1. ACV synthase, encoded by *pcbAB*, condense 3 precursors including L-α-aminoadipic acid, L-cysteine, L-valine to ACV tripeptide. A comprehensively written review on this enzyme was published by Zhang & Demain in 1991 [18]. In *Streptomyces clavuligerus*, Lysine ε-aminotransferase (LAT) is also within the early gene cluster for cephamycin C biosynthesis. Since cephamycin C shares most of the biosynthetic genes with CPC biosynthesis pathway, it has been studied extensively by Demain's group [19–21].

ACV is then cyclized into isopenicillin N (IPN) by IPN synthase encoded by *pcbC*. Conversion from IPN to penicillin N was catalyzed by a two-component epimerization system encoded by *cefD1-cefD2*. *cefEF* encodes a unique bi-functional enzyme, deacetyloxycephalosporin C (DAOC) synthase-hydroxylase which successively transforms penicillin N into DAOC and deacetyl-cephalosporin C (DAC). The last step in CPC biosynthesis is catalyzed by DAC-acetyltransferase (DAC-AT) which was encoded by *cefG*. The crystal structure of DAC-AT has been published. It was found that DAC-AT belongs to α/β hydrolase family based on the formation of DAC-enzyme complex [22]. Among these enzymological steps, *pcbAB*, *cefEF* and *cefG* were considered as the rate-limiting factors in CPC biosynthesis [23].

Recent years, some other regulatory proteins, which have been found important in CPC biosynthesis, as well as their coding genes were discovered. For example, Ac*veA*, a homologue of *veA* from *Aspergillus*, regulates the transcription of all six major CPC biosynthesis genes including *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF* and *cefG*. Disruption of Ac*veA* leads to a dramatic reduction of CPC yield [24]. We also tried overexpression of Ac*veA* and the production of CPC was increased, as expected, by 22.7%. The transcriptions of *pcbC*, *cefEF* and *cefG* were all upreglated [25].

A *cefP* gene located in the early cluster of CPC biosynthesis cluster was just characterized. This gene encodes a transmembrane protein anchored on peroxisome. It regulates the epimerization of IPN to penicillin N catalyzed by CefD1-CefD2 two-component enzyme complex in peroxisome. *cefP* disruptant accumulated IPN and lost CPC production [26]. To compensate the disruption of *cefP*, *cefP* and *cefR* are necessary to be introduced simultaneously. CefR is the repressor of CefT, and stimulate the transcription of *cefEF*. The *cefR* knock out mutant showed a delayed transcription of *cefEF* and accumulation of penicillin N results in reduction of CPC yield [27].

A *cefM* gene was also found downstream of *cefD1*. Disruption of *cefM* accumulates penicillin N but no CPC production at all [28]. It is suggested that CefM may involve in the translocation of penicillin N from peroxisome to cytoplasm. Without *cefM*, cells are unable to transport penicillin N which is epimerized in peroxisome into cytoplasm, where CPC is synthesized.

3. Genetic engineering of *Acremonium chrysogenum*

Acremonium chrysogenum belongs to the family of Filamentous fungi. The techniques for genetic engineering are somehow hard to develop due to its complicate structure of cell wall and the special life cycle. Our lab has started the study of *A. chrysogenum* at molecular level based on some published results from host, transformation, homologous recombination and selection marker of *A. chrysogenum* [29,30].

To introduce exogenous DNA into *A. chrysogenum*, a traditional PEG mediated protoplast transformation method is commonly used [31]. Since we are focusing on high-yield, or industrial strains, which usually have a stronger restriction-modification system than wide-type strain, the traditional transformation method is not efficient enough for introduction of exogenous genes.

Agrobacterium tumefaciens mediated transformation has been widely used in plant genetic engineering, and in some of the Filamentus fungi including *Penicillium chrysogenum* and *Aspergillus nidulans* as well. We have developed an adapted *A. tumefaciens* mediated transformation protocol for *A. chrysogenum*, which has a higher transformation efficiency than the PEG mediated method [32], and more importantly, this protocol can also be applied in *A. chrysogenum* high-yield strain. This is the first report of *A. tumefaciens* mediated *A. chrysogenum* transformation.

A lot of basic research was done to facilitate the genomic DNA extraction [33] and endogenous promoter capture [34] from the chromosome of *A. chrysogenum*. A notable progress is the cloning of *pcbAB-pcbC* bi-directional promoter from the chromosome of *A. chrysogenum* [35]. This enables convenient manipulation of *A. chrysogenum* by introduction of multiple genes afterwards.

Two inducible endogenous promoter P*mir1* [36] and P*xyl1* [37] were identified to be suitable for conditional expression in *A. chrysogenum*. These approaches not only provide the efficient tools for genetic engineering of *A. chrysogenum*, but also make it

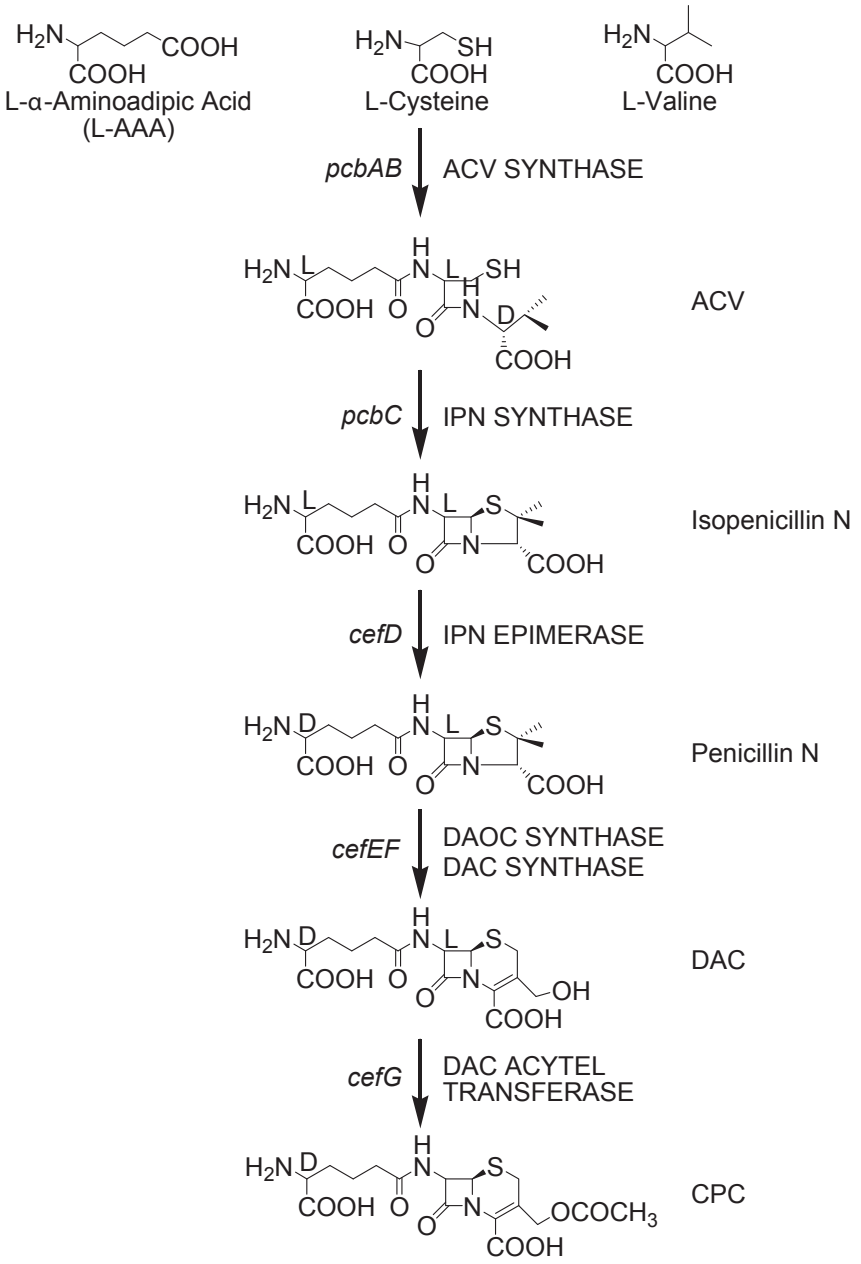

Fig. 1. The biosynthesis pathway of CPC.

possible for selection marker recycling, given the truth that only a few selection markers are applicable in this Filamentus fungus.

The exogenous promoter Sm*xyl* from *Sordaria macrospore* can also be used to construct the selection marker free recombinant mutant by using AcKu70 knock out strain as the recipient, and one step FLP/FRT recombinant system was successfully introduced to facilitate the genetic engineering of *A. chrysogenum*. This process can insert the exogenous gene into the genome of the CPC-producing strain without addition of the antibiotic-resistant gene [38].

Besides the introduction of exogenous genes, disruption and/or silencing of the endogenous genes is also a common strategy for genetic breeding of a certain strain. The recently developed RNA interference (RNAi) technique can be used as an alternative to silence the transcription of target genes instead of homologous recombination. RNAi in *A. chrysogenum* was first published in 2007 [39]. The latest report was silencing of *pcbC* gene in *Penicillium chrysogenum* and *cefEF* gene in *A. chrysogenum* by RNAi [40]. These reports demonstrated the feasibility of RNAi technique in the Filamentous fungi.

We also tried the RNAi technique in *A. chrysogenum*. A plasmid vector containing *cefG* double strand RNA transcription unit was constructed and transformed into CPC-producing *A. chrysogenum*. The *cefG* transcription level in the transformants was measured by quantitative RT-PCR. The *cefG* transcription level decreases to 20% in two mutant strains. Their CPC yield was also found to decrease by 34.6% and 28.8%, respectively [41]. This result demonstrated the feasibility of RNAi application in *A. chrysogenum* and possible, industrial strain. Moreover, this is important for metabolic pathway reconstitution and novel CPC derivatives fermentation in *A. chrysogenum*.

By using the techniques that have been developed, researchers are able to study gene function *in vivo* to elucidate their impact on

CPC production in *A. chrysogenum*. A glutathione reductase coding gene *glrA* was knocked out in *A. chrysogenum*, and the activity of glutathione reductase was disrupted leading to the accumulation of glutathione disulfide. This mutant is sensitive to oxygen stress and produces non-detectable cephalosporins. Overexpression of *glrA* can restore the glutathione reductase activity and the ratio of GSH/GSSR. The growth and CPC production can also be recovered by addition of methionine [42]. Similarly, a thioredoxin reductase-encoding gene Ac*trxR1* was also knocked out and spore germination and hyphal growth were inhibited. However, addition of DL-methionine can restore the delay and CPC production was enhanced, as well as the transcription of *pcbC*, *cefEF* and *cefG* [43].

The fungal nitrogen regulatory GATA zinc-finger protein Ac*areA* was disrupted in *A. chrysogenum* and the mutation causes the reduction of CPC production. Meanwhile, transcriptions of CPC biosynthetic genes including *pcbAB*, *cefD2*, *cefEF* and *cefG* were downregulated. Ac*areA* binds not only to the promoter region of nitrate reductase gene *niaD-niiA*, but also to the bidirectional promoter of *pcbAB-pcbC* [44].

Impaired growth mutant of *A. chrysogenum* was analyzed and a partial deleted putative septation protein encoding gene Ac*sepH* was identified. Absence of *AcsepH* leads to reduction of septation and formation of multinucleate cells in *A. chrysogenum*. Also, conidia and CPC production was significantly decreased along with delay and decreased transcriptions of *pcbC*, *cefD1*, *cefD2* and *cefEF* [45].

Ac*atg1* is a homolog of eukryotic serine/threonine kinase involved in autophagy. Disruption of Ac*atg1* in *A. chrysogenum* significantly reduces the formation of conidia, similar to disruption of Ac*sepH*. However, CPC production was surprisingly enhanced in the Ac*atg1* disruption mutant and PcbC expression was kept at high level all through the fermentation process [46].

Our lab cloned a putative thiazole biosynthesis gene Ac*thi* and found knock out of Ac*thi* in *A. chrysogenum* reduced the intracellular concentration of thiamine and CPC biosynthesis precursor amino acids, while the formation of conidia was also decreased. Overexpression of Ac*thi* can restore the reduction, upregulate the transcriptions of CPC biosynthetic genes and increase the production of CPC [47].

4. Omics study of *Acremonium chrysogenum*

Recent years, researchers have started genetic engineering of *A. chrysogenum* at the omics level by comparing the high yield strain with the low yield strain. Scientists from Russia used qPCR to detect the transcriptional level of CPC biosynthesis related genes in a high-producing strain RNCM F-4081D and the wide-type strain ATCC 11550 [48]. Results revealed that regulatory genes *cefM* and *cefR* were upregulated in the CPC high-producing strain. All biosynthetic genes including *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF* and *cefG* had significantly higher transcriptional levels in RNCM F-4081D strain, which produces 100-fold more CPC than the wide-type strain. Although these results are anticipated, the balanced transcriptions of the biosynthetic genes and regulatory genes could give us some ideas to improve CPC production in *A. chrysogenum*.

We have studied the proteomics [49] and transcriptomics of an industrial CPC-producing strain (high-yield, HY) and the wide-type (WT) strain. According to comparative proteomics results, several significantly differentially expressed proteins including Ac*thi* were identified. Comparative transcriptomics study revealed more information. A total of 4329 genes were identified that have significantly differential transcription levels in which 1737 were upregulated and 2592 were downregulated in HY strain [50]. These differentially expressed genes belong to the pathways of carbohydrate metabolism and energy, defense and virulence mechanisms, CPC biosynthetic precursor amino acids, oxidative stress defenses, and other secondary metabolites.

We also looked at the transcriptional level of some key genes involved in the primary metabolism in *A. chrysogenum*, which haven't been systemically studied as well as its correlation with CPC production to date. Glycolysis and gluconeogenesis, TCA cycle and Glyoxylate cycle, Pentose phosphate pathway and GABA shunt, and serine, lysine, cysteine, valine biosynthesis were studied and five important intermediates including glucose 6-phosphate, 3-phosphoglycerate, pyruvate, malate and succinate were identified. The involved metabolic pathways showed shifted flows which are favorable for switching to CPC production in HY strain [51]. These are very important information for future metabolic engineering of the CPC-producing *A. chrysogenum*.

Without the genomic sequence, omics study gave us the overall profile of the CPC-producing *A. chrysogenum*. Not surprisingly, this important industrial strain was sequenced recently, and the full genomic sequence was publicly revealed [52]. The sequence showed it has about 28.6 Mb in length, and approximately 8901 coding regions. However, this is shorter than the estimation of 32.7 Mb before [53]. Also the sequenced strain is the wide-type ATCC 11550. Lots of gaps are still open in the sequence. This also drew our attentions because there are several genes identified in our transcriptome study were not found in the published sequence, including Ac*thi*.

The published genome also contains the mitochondrial sequence, which was published by other researchers elsewhere. This 27 Kb circular molecule contains 19 coding regions and 26 tRNA genes. Interestingly, all genes are located on one strand and may transcribed in the same direction [54]. Publication of both the chromosomal and mitochondrial sequences, as well as the transcriptome and proteome study will boost the genetic engineering of this important Filamentus fungus.

5. Molecular breeding of *Acremonium chrysogenum*

Among the three rate-limiting enzyme, PcbAB is relatively difficult to manipulate due to its larger coding region. Thus, researchers focus on *cefEF* and *cefG* for molecular breeding of *A. chrysogenum*. Besides, extra copy numbers of *cefT* could increase the yield of CPC in *A. chrysogenum* [55]. And, overexpression of *cefP* and *cefR* in *A. chrysogenum* can decrease the accumulation of penicillin N and promote the yield of CPC by about 50% [27].

The fermentation process of *A. chrysogenum* is an extreme oxygen-consuming procedure. All the rate-limiting enzymes are oxygen-requiring enzymes. The *Vitreoscilla* Hemoglobin (VHb) is very attractable since it's capable of oxygen transmission in oxygen-limiting environment. A recombinant strain bearing VHb can significantly improve the usage of oxygen during the fermentation process and increase the product yield, which has been proved in *Aspergillus* [56]. Introduction of *vgb*, the coding gene for VHb, into *A. chrysogenum* can also maintain a higher specific growth rate and specific production rate resulted in a 4–5 fold higher yield of the mutant strain [57]. Actually, there are many industrial *A. chrysogenum* strains that have recombinant *vgb*.

The earliest report on genetic modification for *A. chrysogenum* was published in 1989, when researchers from Eli Lilly Co. introduced extra copy of *cefEF-cefG* fragment into *A. chrysogenum* resulted in a 15%–40% higher producing strain [58]. This is the first evidence that molecular breeding can be a powerful tool in strain improvement of *A. chrysogenum*.

Although controlled by the same bi-directional promoter, the transcription levels of *cefEF* and *cefG* showed a huge difference according to RT-PCR. The transcription of *cefG* is much lower than that of *cefEF*. This leads to the accumulation of DAC in the metabolites since they can not be efficiently transformed into CPC. As a

matter of fact, CPC/DAC ratio is a quality control parameter in the industrial production of CPC fermentation. Thus, introduction of extra copy numbers of *cefG* produced an engineering strain whose CPC yield is 3-fold-higher than the parental strain [59].

There is another report on introduction of *cefT* into *A. chrysogenum*, and the resulting mutant doubled the CPC yield [60]. This could attribute to the enhancement of CefT, the efflux pump protein, so that the feedback inhibition *in vivo* triggered by the fermentation product was attenuated, resulted in an elevated product yield.

Using molecular breeding technology, some CPC derivatives can be directly produced by engineering *A. chrysogenum* fermentation. For example, by disruption of *cefEF* and introduction of *cefE* originated from *Streptomyces clavuligerus*, a novel DAOC producing strain was obtained. Followed by two enzymatic transformations, the industrial important 7-ADCA can be produced [61]. By introduction of two coding genes simultaneously into *A. chrysogenum* for the enzymes used in industrial production of 7-ACA by immobilized enzymatic transformation, the engineering strain can produce 7-ACA by fermentation [62].

Only one enzyme (DAOC/DAC synthase) is responsible for synthesis of deacetylcephalosporin C (DAC) from penicillin N. The coding gene for DAOC/DAC synthase is *cefEF* in *A. chrysogenum*. However, in *Streptomyces clavuligerus* (cephamycin C producer), there are two enzymes responsible for this two step conversion encoded by *cefE* and *cefF*. Introduction of *cefF* (coding for DAOC hydroxylase) into *A. chrysogenum* can significantly decrease the DAOC/CPC ratio given that DAOC is a major impurity in the fermentation of CPC production [63].

There is another interesting research for the molecular breeding of *A. chrysogenum* in a different way. As we mentioned above, CefD1-CefD2 is a two-component enzyme complex that transform IPN into penicillin N by an epimerization system located in peroxisome. *cefD1-cefD2* block mutant lacking this epimerization system accumulated a large amount of IPN to more than 650 μg/mL, almost the total relative CPC yield. The unstable IPN, which never has been purified before, could be purified by several steps of chromatography in this mutant [64]. Characterization of its half-life and stability under variety conditions can greatly help us for the investigation of IPN.

Genome shuffling [65] is a powerful tool for strain improvement especially when lacking the genomic sequence of the target strain. We have proved that this technique is also effective in *A. chrysogenum*. By genetic engineering, we have constructed a strain of *A. chrysogenum* that can directly produce 7-ACA by fermentation, however, the yield is still low [66]. With the help of genome shuffling, the yield of 7-ACA has been increased by 6.5 fold by two round of screening [67]. This makes it potential possible to manufacture 7-ACA, the important β-lactam intermediate, by one-step *A. chrysogenum* fermentation instead of the current two-step process which consist of fermentation of *A. chrysogenum* plus the enzymatic transformation of CPC [68].

It's worth noting that all of the above genetic breeding reports were on the background of an *A. chrysogenum* wide type strain, whose CPC yield is only around 1 mg/mL, far less than the industrial production level, which is around 15–20 mg/mL in flask and 35–40 mg/mL in fermentor. Although some great achievements were obtained in improvement of *A. chrysogenum* fermentation and modification of metabolic products, those achievements are still far away from application in industry.

6. Genetic engineering of industrial *Acremonium chrysogenum*

Our research is focusing on molecular breeding of *A. chrysogenum* high-yield and/or industrial strains. We introduced different combinations of *cefG*/*cefEF*/*cefT*/*vgb* genes into CPC high-producing strain and found that an extra copy of *cefG* has significant positive effect on CPC fermentation level. Since random integration was occurred in *A. chrysogenum*, different transformants with *cefG* introduction showed different elevated levels, with 100% promotion at most. An extra copy of *vgb* gene that had been under directed evolution and had elevated bioactivity [69] also displayed a significant improvement up to 30% more of CPC yield. Meanwhile, introduction of *cefEF* and *cefT* has no obvious effect on CPC production in high-yield strain [70]. This revealed the apparent discrepancy between the genetic background of wide-type strain and high-yield strain, and also suggested that endogenous *cefEF* and *cefT* may already achieve high bioactivity after several rounds of mutagenesis breeding that a high-yield strain usually undertaken.

We then applied this achievement to a CPC industrial strain. Although we couldn't double the CPC yield, we did obtain an engineering strain whose CPC yield was increased by 20%, which has a promising industrialization potential. See Table 1 for a complete list of genes that have been manipulated in *A. chrysogenum* (low or high producers) and their influence on CPC yield.

The fermentation product of *A. chrysogenum*, CPC, is the major resource for industrial manufacturing of 7-ACA, the important intermediate of a large variety of cephalosporins antibiotics. A traditional producing route of 7-ACA is the chemical semi-biosynthesis. Up to date, the more environmental-friendly biotransformation has been widely used in industry. Two step transformation using DAO (D-amino acid oxidase) and GL-7-ACA acylase dominates the market for decades [71], more recently one step transformation from CPC to 7-ACA by CPC acylase has become available [72], although, the substrate specificity of CPC acylase still need improved [73].

Whether two-step or one-step, fermentation of CPC is the prerequisite followed by enzymatic biotransformation *in vitro*. We are thinking of introducing CPC acylase gene into *A. chrysogenum* to construct the engineering strain that can produce 7-ACA directly by fermentation, a breakthrough in the production of 7-ACA.

A CPC acylase gene was designed according to the codon bias of *A. chrysogenum* and introduced into an industrial strain. Our result showed that this CPC acylase was expressed in *A. chrysogenum* with bioactivity. The recombinant acylase can transform the original

Table 1
Manipulated genes in CPC low/high producers and their influence on yield.

Gene	Manipulation	Producer	CPC yield	Reference
Ac*veA*	Knock out	low	decrease	[24]
Ac*veA*	overexpression	low	increase	[25]
cefP	Knock out	low	None	[26]
cefR	Knock out	low	decrease	[27]
cefP/*cefR*	overexpression	low	increase	[27]
cefM	Knock out	Low	decrease	[28]
cefEF	Knock down	low	decrease	[40]
cefG	Knock down	low	decrease	[41]
glrA	Knock out	low	none	[42]
Ac*trxR1*	Knock out	low	delay	[43]
Ac*areA*	Knock out	low	decrease	[44]
Ac*sepH*	Knock out	low	decrease	[45]
Ac*atg1*	Knock out	low	increase	[46]
Ac*thi*	Knock down	low/high	decrease	[47]
Ac*thi*	overexpression	low/high	increase	[47]
cefT	overexpression	low	increase	[55] [60]
vgb	overexpression	low	increase	[57]
cefEF-cefG	overexpression	high	increase	[58]
cefG	overexpression	low	increase	[59]
cefG/vgb	overexpression	low/high	increase	[70]

product CPC into 7-ACA *in vivo*, which makes the engineering strain capable of direct fermentation of 7-ACA. Based on enzymological profiles of CPC acylase *in vitro*, we performed a preliminary optimization of medium composition and culture condition. 7-ACA yield was increased significantly in this engineering strain with as least 30% of the fermented CPC was transformed into 7-ACA [74]. We believe this *in vivo* conversion can be more effective if a stronger transcription cassette can be introduced and copy number can be increased. With the incorporation of traditional breeding technology, *in vivo* conversion will be realized in industry.

7. Perspectives

The biosynthesis of CPC in *A. chrysogenum* has been investigated thoroughly. However, the mechanism of its regulation as well as the precursor biosynthesis in primary metabolism is still unclear [75]. Full sequence of *A. chrysogenum* has been published, plus there are more than 10 species belongs to Filamentous fungi have been sequenced [76]. However, gaps need to be filled, and all genes need to be annotated and studied to understand their *in vivo* functions. With the information of the genes that have been published and studied, we can manipulate the genome of *A. chrysogenum* more efficiently. CRISPR/Cas9 is a powerful tool for genome editing in animals [77] and mammalian cells [78] since its discovery. Currently, this technique has been used in microorganisms including *E. coli* [79], *Streptomyces* [80] and Yeast [81]. Although Filamentous fungi is relative difficult to genetically modify, we are trying this system in *A. chrysogenum* in our lab. There are also some publications on genome editing of Filamentous fungi such as *Aspergillus* [82–84], *Pyricularia* [85], *Trichoderma* [86] and *Penicillium* [87] in the recent two years. Thus, we believe it is possible to edit the genome of *A. chrysogenum* by the newly developed system.

By identifying differentially expressed proteins during CPC fermentation in wide-type strain and high-yield strain, a comprehensive regulation system of *A. chrysogenum* fermentation may be proposed based on the popular theory of metabolic engineering and systems biology [88] because omics study has shown to be a powerful tool to study the metabolic pathway from a systemic scope. A study on *Penicillin chrysogenum* revealed that 950 proteins involved in precursor biosynthesis, stress response and pentose phosphate pathway were found to be related to the fermentation yield in three penicillin-producing strains [89]. We have identified several differentially expressed proteins that are associated with several metabolic pathways, especially those involved in primary metabolism of *A. chrysogenum*. Study of these genes along with their involved pathways is in progress and can help us better understand how the primary metabolism will influence the CPC biosynthesis. And we believe this should incorporate metabolomics study [90] that helps us explain from the transcription and expression to metabolism.

Dr. Demain opened up a new era fifty years ago. Now, with the help of modern molecular techniques and more powerful tools, we believe that genetic modified *Acremonium chrysogenum* will play an important role in the industrial production of CPC and its derivatives.

References

[1] Demain AL, Elander RP. The beta-lactam antibiotics: past, present, and future. Antonie Leeuwenhoek 1999;75:5–19.
[2] Demain AL. Biosynthesis of cephalosporin C and its relation to penicillin formation. Trans N Y Acad Sci 1963;25:731–40.
[3] Demain AL, Newkirk JF. Biosynthesis of cephalosporin C. Appl Microbiol 1962;10:321–5.
[4] Demain AL, Newkirk JF, Hendlin D. Effect of methionine, norleucine, and lysine derivatives on cephalosporin C formation in chemically defined media. J Bacteriol 1963;85:339–44.
[5] Demain AL. L-Valine: a precursor of cephalosporin C. Biochem Biophy Res Commun 1963;10:45–8.
[6] Ellaiah P, Adinarayana K, Chand GM, Subramanyam GS, Srinivasulu B. Strain improvement studies for cephalosporin C production by *Cephalosporium acremonium*. Pharmazie 2002;57:489–90.
[7] Ellaiah P, Kumar JP, Saisha V, Sumitra JJ, Vaishali P. Strain improvement studies on production of cephalosporin C from *Acremonium chrysogenum* ATCC 48272. Hindustan Antibiot Bull 2003;45–46:11–5.
[8] Lee MS, Lim JS, Kim CH, Oh KK, Yang DR, Kim SW. Enhancement of cephalosporin C production by cultivation of *Cephalosporium acremonium* M25 using a mixture of inocula. Lett Appl Microbiol 2001;32:402–6.
[9] Shin HY, Lee JY, Park C, Kim SW. Utilization of glycerol as cysteine and carbon sources for cephalosporin C production by *Acremonium chrysogenum* M35 in methionine-unsupplemented culture. J Biotechnol 2011;151:363–8.
[10] Shin HY, Lee JY, Choi HS, Lee JH, Kim SW. Production of cephalosporin C using crude glycerol in fed-batch culture of *Acremonium chrysogenum* M35. J Microbiol 2011;49:753–8.
[11] Lopez-Calleja AC, Cuadra T, Barrios-Gonzalez J, Fierro F, Fernandez FJ. Solid-state and submerged fermentations show different gene expression profiles in cephalosporin C production by *Acremonium chrysogenum*. J Mol Microbiol Biotechnol 2012;22:126–34.
[12] Drew SW, Demain AL. Methionine control of cephalosporin C formation. Biotechnol Bioeng 1973;15:743–54.
[13] Martin JF, Demain AL. Unraveling the methionine-cephalosporin puzzle in *Acremonium chrysogenum*. Trends Biotechnol 2002;20:502–7.
[14] Demain AL, Zhang J. Cephalosporin C production by *Cephalosporium acremonium*: the methionine story. Crit Rev Biotechnol 1998;18:283–94.
[15] Gutierrez S, Diez B, Montenegro E, Martin JF. Characterization of the *Cephalosporium acremonium pcbAB* gene encoding alpha-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: linkage to the *pcbC* gene as a cluster of early cephalosporin biosynthetic genes and evidence of multiple functional domains. J Bacteriol 1991;173:2354–65.
[16] Martin JF, Ullan RV, Casqueiro J. Novel genes involved in cephalosporin biosynthesis: the three-component isopenicillin N epimerase system. Adv Biochem Eng Biotechnol 2004;88:91–109.
[17] Gutierrez S, Velasco J, Fernandez FJ, Martin JF. The *cefG* gene of *Cephalosporium acremonium* is linked to the *cefEF* gene and encodes a deacetylcephalosporin C acetyltransferase closely related to homoserine O-acetyltransferase. J Bacteriol 1992;174:3056–64.
[18] Zhang J, Demain AL. Regulation of ACV synthetase in penicillin- and cephalosporin-producing microorganisms. Biotechnol Adv 1991;9:623–41.
[19] Rius N, Demain AL. Lysine epsilon-aminotransferase, the initial enzyme of cephalosporin biosynthesis in actinomycetes. J Microbiol Biotechnol 1997;7:95–100.
[20] Yu H, Serpe E, Romero J, Coque JJ, Maeda K, Oelgeschlager M, et al. Possible involvement of the lysine epsilon-aminotransferase gene (*lat*) in the expression of the genes encoding ACV synthetase (*pcbAB*) and isopenicillin N synthase (*pcbC*) in *Streptomyces clavuligerus*. Microbiology 1994;140(Pt 12):3367–77.
[21] Demain AL, Piret JM, Yu H, Coque JJ, Liras P, Martin JF. Interdependence of gene expression for early steps of cephalosporin synthesis in *Streptomyces clavuligerus*. Ann N Y Acad Sci 1994;721:117–22.
[22] Lejon S, Ellis J, Valegard K. The last step in cephalosporin C formation revealed: crystal structures of deacetylcephalosporin C acetyltransferase from *Acremonium chrysogenum* in complexes with reaction intermediates. J Mol Biol 2008;377:935–44.
[23] Brakhage AA, Thon M, Sprote P, Scharf DH, Al-Abdallah Q, Wolke SM, et al. Aspects on evolution of fungal beta-lactam biosynthesis gene clusters and recruitment of trans-acting factors. Phytochemistry 2009;70:1801–11.
[24] Dreyer J, Eichhorn H, Friedlin E, Kurnsteiner H, Kuck U. A homologue of the *Aspergillus* velvet gene regulates both cephalosporin C biosynthesis and hyphal fragmentation in *Acremonium chrysogenum*. Appl Environ Microbiol 2007;73:3412–22.
[25] Gong G, Zhang W, Hu Y, Zhu B. Overexpression Acv*eA* in *A. chrysogenum* to improve CPC production. Chin J Pharm 2014;45:719–23.
[26] Ullan RV, Teijeira F, Guerra SM, Vaca I, Martin JF. Characterization of a novel peroxisome membrane protein essential for conversion of isopenicillin N into cephalosporin C. Biochem J 2010;432:227–36.
[27] Teijeira F, Ullan RV, Fernandez-Lafuente R, Martin JF. CefR modulates transporters of beta-lactam intermediates preventing the loss of penicillins to the broth and increases cephalosporin production in *Acremonium chrysogenum*. Metab Eng 2011. http://dx.doi.org/10.1016/j.ymben.2011.06.004.
[28] Teijeira F, Ullan RV, Guerra SM, Garcia-Estrada C, Vaca I, Martin JF. The transporter CefM involved in translocation of biosynthetic intermediates is essential for cephalosporin production. Biochem J 2009;418:113–24.
[29] Kuck U, Hoff B. New tools for the genetic manipulation of filamentous fungi. Appl Microbiol Biotechnol 2010;86:51–62.
[30] Meyer V. Genetic engineering of filamentous fungi–progress, obstacles and future trends. Biotechnol Adv 2008;26:177–85.
[31] Skatrud PL, Queener SW, Carr LG, Fisher DL. Efficient integrative transformation of *Cephalosporium acremonium*. Curr Genet 1987;12:337–48.
[32] Xu W, Zhu C, Zhu B. An efficient and stable method for the transformation of heterogeneous genes into *Cephalosporium acremonium* mediated by *Agrobacterium tumefaciens*. J Microbiol Biotechnol 2005;15:683–8.
[33] Xu W, Zhu C, Zhu B, Yao X. A new method for isolation of chromosomal DNA

from filamentous fungus *Cephalosporium acremonium*. J Shenyang Pharm Univ 2004;21. 226–229,236.
[34] Zhang P, Zhu C, Zhu B, Zhao W. A convenient method to select DNA fragment of *Cephalosporium acremonium* with promoter function. Microbiol China 2004;31:97–100.
[35] Zhang P, Zhu C, Zhu B. Cloning of bidirectional *pcb*AB-*pcb*C promoter region from *Cephalosporium acremonium* and its application. Acta Microbiol Sin 2004;44:255–7.
[36] Gsaller F, Blatzer M, Abt B, Schrettl M, Lindner H, Haas H. The first promoter for conditional gene expression in *Acremonium chrysogenum*: iron starvation-inducible *mir1*(P). J Biotechnol 2013;163:77–80.
[37] Blatzer M, Gsaller F, Abt B, Schrettl M, Specht T, Haas H. An endogenous promoter for conditional gene expression in *Acremonium chrysogenum*: the xylan and xylose inducible promoter *xyl1*(P. J Biotechnol 2014;169:82–6.
[38] Bloemendal S, Loper D, Terfehr D, Kopke K, Kluge J, Teichert I, et al. Tools for advanced and targeted genetic manipulation of the beta-lactam antibiotic producer *Acremonium chrysogenum*. J Biotechnol 2014;169:51–62.
[39] Janus D, Hoff B, Hofmann E, Kuck U. An efficient fungal RNA-silencing system using the DsRed reporter gene. Appl Environ Microbiol 2007;73:962–70.
[40] Ullan RV, Godio RP, Teijeira F, Vaca I, Garcia-Estrada C, Feltrer R, et al. RNA-silencing in *Penicillium chrysogenum* and *Acremonium chrysogenum*: validation studies using beta-lactam genes expression. J Microbiol Methods 2008;75:209–18.
[41] Gong G, Liu Y, Hu Y, Zhu C, Zhu B. Down-regulation of *cefG* gene transcription in an industrial strain of *Acremonium chrysogenum* by RNA interference. Biotechnol Bull 2010;10:193–7.
[42] Long LK, Yang J, An Y, Liu G. Disruption of a glutathione reductase encoding gene in *Acremonium chrysogenum* leads to reduction of its growth, cephalosporin production and antioxidative ability which is recovered by exogenous methionine. Fungal Genet Biol FG B 2012;49:114–22.
[43] Liu L, Long LK, An Y, Yang J, Xu X, Hu CH, et al. The thioredoxin reductase-encoding gene A*trxR1* is involved in the cephalosporin C production of *Acremonium chrysogenum* in methionine-supplemented medium. Appl Microbiol Biotechnol 2013;97:2551–62.
[44] Li J, Pan Y, Liu G. Disruption of the nitrogen regulatory gene A*careA* in *Acremonium chrysogenum* leads to reduction of cephalosporin production and repression of nitrogen metabolism. Fungal Genet Biol FG B 2013;61:69–79.
[45] Long LK, Wang Y, Yang J, Xu X, Liu G. A septation related gene A*csepH* in *Acremonium chrysogenum* is involved in the cellular differentiation and cephalosporin production. Fungal Genet Biol FG B 2013;50:11–20.
[46] Wang H, Pan Y, Hu P, Zhu Y, Li J, Jiang X, et al. The autophagy-related gene Acatg1 is involved in conidiation and cephalosporin production in *Acremonium chrysogenum*. Fungal Genet Biol FG B 2014;69:65–74.
[47] Liu Y, Zhang W, Xie L, Liu H, Gong G, Zhu B, et al. A*cthi*, a thiazole biosynthesis enzyme, is essential for thiamine biosynthesis and CPC production in *Acremonium chrysogenum*. Microb Cell Fact 2015;14:50.
[48] Dumina MV, Zhgun AA, Novak MI, Domratcheva AG, Petukhov DV, Dzhavakhiya VV, et al. Comparative gene expression profiling reveals key changes in expression levels of cephalosporin C biosynthesis and transport genes between low and high-producing strains of *Acremonium chrysogenum*. World J Microbiol Biotechnol 2014;30:2933–41.
[49] Liu H, Liu Y, Xie L, Zhu B, Hu Y. Proteome preparation for *Acremonium chrysogenum*. Chin J Pharm 2011;42:412–5.
[50] Liu Y, Xie L, Gong G, Zhang W, Zhu B, Hu Y. De novo comparative transcriptome analysis of *Acremonium chrysogenum*: high-yield and wild-type strains of cephalosporin C producer. PloS One 2014;9:e104542.
[51] Han S, Liu Y, Xie L, Zhu B, Hu Y. Comparative expression profiling of genes involved in primary metabolism in high-yield and wild-type strains of *Acremonium chrysogenum*. Antonie Leeuwenhoek 2016;109:357–69.
[52] Terfehr D, Dahlmann TA, Specht T, Zadra I, Kurnsteiner H, Kuck U. Genome sequence and annotation of *Acremonium chrysogenum*, producer of the beta-lactam antibiotic cephalosporin C. Genome Announc 2014;2.
[53] Walz M, Kuck U. Polymorphic karyotypes in related *Acremonium* strains. Curr Genet 1991;19:73–6.
[54] Eldarov MA, Mardanov AV, Beletsky AV, Dumina MV, Ravin NV, Skryabin KG. Complete mitochondrial genome of the cephalosporin-producing fungus *Acremonium chrysogenum*. Mitochondrial DNA 2015;26:943–4.
[55] Nijland JG, Kovalchuk A, van den Berg MA, Bovenberg RA, Driessen AJ. Expression of the transporter encoded by the *cefT* gene of *Acremonium chrysogenum* increases cephalosporin production in *Penicillium chrysogenum*. Fungal Genet Biol 2008;45:1415–21.
[56] Lin YH, Li YF, Huang MC, Tsai YC. Intracellular expression of *Vitreoscilla* hemoglobin in *Aspergillus terreus* to alleviate the effect of a short break in aeration during culture. Biotechnol Lett 2004;26:1067–72.
[57] DeModena JA, Gutierrez S, Velasco J, Fernandez FJ, Fachini RA, Galazzo JL, et al. The production of cephalosporin C by *Acremonium chrysogenum* is improved by the intracellular expression of a bacterial hemoglobin. Biotechnology 1993;11:926–9.
[58] Skatrud P, Tietz A, Ingolia T, Cantwell C, Fisher D, Chapman J, et al. Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. Biotechnology 1989;7:477–85.
[59] Gutierrez S, Velasco J, Marcos AT, Fernandez FJ, Fierro F, Barredo JL, et al. Expression of the *cefG* gene is limiting for cephalosporin biosynthesis in *Acremonium chrysogenum*. Appl Microbiol Biotechnol 1997;48:606–14.
[60] Ullan RV, Liu G, Casqueiro J, Gutierrez S, Banuelos O, Martin JF. The *cefT* gene of *Acremonium chrysogenum* C10 encodes a putative multidrug efflux pump protein that significantly increases cephalosporin C production. Mol Genet Genomics 2002;267:673–83.
[61] Velasco J, Luis Adrio J, Angel Moreno M, Diez B, Soler G, Barredo JL. Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of *Acremonium chrysogenum*. Nat Biotechnol 2000;18:857–61.
[62] Isogai T, Fukagawa M, Aramori I, Iwami M, Kojo H, Ono T, et al. Construction of a 7-aminocephalosporanic acid (7ACA) biosynthetic operon and direct production of 7ACA in *Acremonium chrysogenum*. Biotechnol N Y 1991;9:188–91.
[63] An Y, Dong H, Liu G. Expression of *cefF* significantly decreased deacetoxycephalosporin C formation during cephalosporin C production in *Acremonium chrysogenum*. J Ind Microbiol Biotechnol 2012;39:269–74.
[64] Vaca I, Casqueiro J, Ullan RV, Rumbero A, Chavez R, Martin JF. A preparative method for the purification of isopenicillin N from genetically blocked *Acremonium chrysogenum* strain TD189: studies on the degradation kenetics and storage conditions. J Antibiot 2011;64:447–51.
[65] Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WP, del Cardayre SB. Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 2002;415:644–6.
[66] Hu Y, Liu Y, Zhu B. Construction of an engineering *Acremonium chrysogenum* for fermentation of 7-ACA. Chin Bull Life Sci 2011;23:912–6.
[67] Liu C, Liu Y, Xie L, Hu Y. Improvement of 7-ACA producing strain by genomic shuffling. Chin J Pharm 2013;44:440–5.
[68] Conlon HD, Baqai J, Baker K, Shen YQ, Wong BL, Noiles R, et al. Two-step immobilized enzyme conversion of cephalosporin C to 7-aminocephalosporanic acid. Biotechnol Bioeng 1995;46:510–3.
[69] Yuan N, Hu Y, Zhu C, Zhu B. DNA shuffling of *Vitreoscilla* hemoglobin. Chin Biotechnol 2006;26:14–9.
[70] Liu Y, Gong G, Xie L, Yuan N, Zhu C, Zhu B, et al. Improvement of cephalosporin C production by recombinant DNA integration in *Acremonium chrysogenum*. Mol Biotechnol 2010;44:101–9.
[71] Conlon HD, Baqai J, Baker K, Shen YQ, Wong BL, Noiles R, et al. Two-step immobilized enzyme conversion of cephalosporin C to 7-aminocephalosporanic acid. Biotechnol Bioeng 1995;46:510–3.
[72] Conti G, Pollegioni L, Molla G, Rosini E. Strategic manipulation of an industrial biocatalyst–evolution of a cephalosporin C acylase. FEBS J 2014;281:2443–55.
[73] Sonawane VC. Enzymatic modifications of cephalosporins by cephalosporin acylase and other enzymes. Crit Rev Biotechnol 2006;26:95–120.
[74] Liu Y, Gong G, Zhu C, Zhu B, Hu Y. Environmentally safe production of 7-ACA by recombinant *Acremonium chrysogenum*. Curr Microbiol 2010;61:609–14.
[75] Schmitt EK, Hoff B, Kuck U. Regulation of cephalosporin biosynthesis. Adv Biochem Eng Biotechnol 2004;88:1–43.
[76] Jones MG. The first filamentous fungal genome sequences: *Aspergillus* leads the way for essential everyday resources or dusty museum specimens? Microbiology 2007;153:1–6.
[77] Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 2013;153:910–8.
[78] Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339:819–23.
[79] Li Y, Lin Z, Huang C, Zhang Y, Wang Z, Tang YJ, et al. Metabolic engineering of Escherichia coli using CRISPR-Cas9 meditated genome editing. Metab Eng 2015;31:13–21.
[80] Cobb RE, Wang Y, Zhao H. High-efficiency multiplex genome editing of Streptomyces species using an engineered CRISPR/Cas system. ACS Synth Biol 2015;4:723–8.
[81] DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res 2013;41:4336–43.
[82] Zhang C, Meng X, Wei X, Lu L. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in Aspergillus fumigatus. Fungal Genet Biol FG B 2016;86:47–57.
[83] Katayama T, Tanaka Y, Okabe T, Nakamura H, Fujii W, Kitamoto K, et al. Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus Aspergillus oryzae. Biotechnol Lett 2016;38:637–42.
[84] Nodvig CS, Nielsen JB, Kogle ME, Mortensen UH. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PloS One 2015;10:e0133085.
[85] Arazoe T, Miyoshi K, Yamato T, Ogawa T, Ohsato S, Arie T, et al. Tailor-made CRISPR/Cas system for highly efficient targeted gene replacement in the rice blast fungus. Biotechnol Bioeng 2015;112:2543–9.
[86] Liu R, Chen L, Jiang Y, Zhou Z, Zou G. Efficient genome editing in filamentous fungus Trichoderma reesei using the CRISPR/Cas9 system. Cell Discov 2015;1:15007.
[87] Pohl C, Kiel JA, Driessen AJ, Bovenberg RA, Nygard Y. CRISPR/Cas9 based genome editing of Penicillium chrysogenum. ACS Synth Biol 2016;5:754–64.
[88] Thykaer J, Nielsen J. Metabolic engineering of beta-lactam production. Metab Eng 2003;5:56–69.
[89] Jami MS, Barreiro C, Garcia-Estrada C, Martin JF. Proteome analysis of the penicillin producer *Penicillium chrysogenum*: characterization of protein changes during the industrial strain improvement. Mol Cell Proteom 2010;9:1182–98.
[90] Gummer JP, Krill C, Du Fall L, Waters OD, Trengove RD, Oliver RP, et al. Metabolomics protocols for filamentous fungi. Methods Mol Biol 2012;835:237–54.