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

Youjia Hu*, Baoquan Zhu

China State Institute of Pharmaceutical Industry, Shanghai 201203, China

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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).

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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