

Expression of *cefF* significantly decreased deacetoxycephalosporin C formation during cephalosporin C production in *Acremonium chrysogenum*

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Abstract Deacetoxycephalosporin C (DAOC) is not only the precursor but also one of the by-products during cephalosporin C (CPC) biosynthesis. One enzyme (DAOC/DAC synthase) is responsible for the two-step conversion of penicillin N into deacetylcephalosporin C (DAC) in *Acremonium chrysogenum*, while two enzymes (DAOC synthase and DAOC hydroxylase) were involved in this reaction in *Streptomyces clavuligerus* and *Amycolatopsis lactamdurans* (*Nocardia lactamdurans*). In this study, the DAOC hydroxylase gene *cefF* was cloned from *Streptomyces clavuligerus* and introduced into *Acremonium chrysogenum* through *Agrobacterium tumefaciens*-mediated transformation. When *cefF* was expressed under the promoter of *pcbC*, the ratio of DAOC/CPC in the fermentation broth significantly decreased. These results suggested that introduction of *cefF* could function quite well in *Acremonium chrysogenum* and successfully reduce the content of DAOC in the CPC fermentation broth. This work offered a practical way to improve the CPC purification and reduce its production cost.

Keywords *cefF* · *Acremonium chrysogenum* · DAOC/CPC ratio

Introduction

Cephalosporin C (CPC), a beta-lactam antibiotic isolated in 1956 from *Acremonium chrysogenum* (formerly *Cephalosporium acremonium*), has been successfully applied to

manufacturing clinical drugs against human infections caused by penicillinase-producing bacteria for the last 50 years [1, 24]. With the development of the pharmaceutical industry, the natural product CPC can be chemically synthesized into more active derivatives, which are less susceptible to penicillinase. 7-amino cephalosporanic acid (7-ACA), one of the CPC derivatives that shares 66% of commercial cephalosporins production, is chemically synthesized by hydrolysis of the nucleus of CPC [23]. During this process, hydroxymethyl-7-aminocephalosporanic acid (7-ADCA) is also synthesized as a by-product and takes a concentration of 1–2%, which causes the impurity of the 7-ACA production [6].

The cephalosporin C biosynthetic pathway in *A. chrysogenum* has been well characterized both biochemically and genetically [11–14, 22, 25, 30, 31]. In this pathway, penicillin N is converted to DAOC (deacetoxycephalosporin C) then to DAC (deacetylcephalosporin C) by one expandase/hydroxylase enzyme [11]. However, the DAOC could not be converted to DAC completely and a small amount of it accumulates in the fermentation broth and later is converted to 7-ADCA when the CPC is hydrolyzed to 7-ACA.

A similar CPC biosynthetic pathway is demonstrated in cephamycin (which is sometimes classified as cephalosporin) producing strain *Streptomyces clavuligerus* and *Amycolatopsis lactamdurans* (*Nocardia lactamdurans*) [20]. However, these reactions from penicillin N to DAC were catalyzed by separate enzymes in *S. clavuligerus* and *N. lactamdurans* [7, 19]. The *A. chrysogenum* expandase/hydroxylase gene (*cefEF*) and the *S. clavuligerus* hydroxylase gene (*cefF*) have been cloned and sequenced, their amino acid sequences show a high degree of similarity [19, 25]. Enzymatic study of the *S. clavuligerus* hydroxylase (CefF) also confirmed the substrate specificity to DAOC [3]. To

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reduce the contamination of DAOC, an attempt of adding an extra copy of *cefEF* gene in *A. chrysogenum* made a 40–70% reduction in the DAOC level [6]. In this report, we have demonstrated that the *S. clavuligerus* hydroxylase gene (*cefF*) could function quite well in *A. chrysogenum* and successfully reduced the content of DAOC in the CPC fermentation broth. This work also showed a great possibility to reduce the cost of CPC purification by improving the industrial strain through this way.

Materials and methods

Microbial strains and media

Escherichia coli TOP10 was purchased from Transgen (Beijing, China) and used for plasmid construction. The cephamycin-producing strain *Streptomyces clavuligerus* CGMCC4.1611 was obtained from China General Microbiological Culture Collection Center (CGMCC). *Micrococcus luteus* CGMCC 1.1848, a β -lactam antibiotics-sensitive strain, was used as the indicator. The wild-type strain *Acremonium chrysogenum* CGMCC3.3795 was used as a host for DNA transformation and for producing cephalosporin C. *Agrobacterium tumefaciens* (updated scientific name: *Rhizobium radiobacter*) AGL-1 was provided by Professor Xingzhong Liu (Institute of Microbiology, CAS).

Escherichia coli and *A. tumefaciens* were grown in Luria broth (1% sodium chloride, 0.5% yeast extract, and 1% tryptone) or Luria broth agar. MM/IM/CM media were used in *A. tumefaciens*-mediated transformation [4, 17]. To isolate the genomic DNA, *S. clavuligerus* was grown in liquid TSB medium (Becton–Dickinson, Franklin Lakes, NJ) in a shake flask at 28°C for 2 days. *A. chrysogenum* was incubated on TSA agar (1.7% tryptone, 0.3% tryptone soya broth, 0.25% glucose, 0.5% sodium chloride, 0.25% K_2HPO_4 , and 1.5% agar) for growth. For sporulation, *A. chrysogenum* was cultured in LPE agar (0.1% glucose, 0.2% yeast extract, 0.15% sodium chloride, 1% calcium chloride, pH6.8, and 2.5% agar) at 28°C for 7 days. For fermentation, five plates of spores were collected and suspended in 2 ml of distilled water and then the spores were inoculated with 100 ml of seed medium in a 500-ml flask on a 250-rpm rotary shaker at 25°C for 2 days [28]. Then, 10 ml of seed culture was used to inoculate 100 ml of defined production medium (M DFA) in a 500-ml shake flask [28]. These cultures (duplicate) were incubated in a rotary shaker at 25°C for 7 days. Production of β -lactam antibiotics was measured by bioassay against *M. luteus* CGMCC 1.1848.

Plasmid construction

Primers 5'-gactagtGTGGATGGCACCTTTTGGG-3' and 5'-cggcgcgccGGTGACGGTTTGTCTGCC-3' (*SpeI* and

AscI sites were underlined) were used to amplify the promoter region (1,188 bps) of *pcbC* using the genomic DNA of *A. chrysogenum* as a template. Primers 5'-aggcgcgccCGAAGGAGCAGGGGAACA-3' and 5'-ccctgcaggCGGCTTGAATGCAACGAC-3' (*AscI* and *SbfI* sites were underlined) were used to amplify *cefF* (1,091 bps) using the genomic DNA of *S. clavuligerus* as a template. Those amplified DNA fragments were ligated into the cloning vector pEASY-Blunt (Transgen, Beijing) and sequenced (Invitrogen, Shanghai) to verify their accuracy. Then, the DNA fragments were digested with the designed enzymes and ligated into the corresponding sites of pAg1-H3 [32] which was previously digested by *SpeI/SbfI* to generate the plasmid pAg1-H3-*cefF*.

Agrobacterium tumefaciens-mediated transformation

The plasmid pAg1-H3-*cefF* was introduced into the competent cells of *A. tumefaciens* AGL-1 via heat shock transformation [10, 15, 16]. One transformant was selected randomly and grown in MM at 28°C for 2 days at 250 rpm. The cultured cells were diluted to 0.15 of OD₆₀₀ in IM and incubated at 28°C for 6 h (OD₆₀₀ reaches 0.6) at 250 rpm. Mix 100 μ l of the cultured cells with 100- μ l suspensions of dispersed *A. chrysogenum* spores (1×10^7 spores per ml) and spread the mixture on CM agar plate. The mixture was incubated at 25°C in the dark for 3 days and transferred to a TSA agar plate supplemented with 50 μ g/ml hygromycin B and 200 μ M of cefotaxime. Colonies of hygromycin B-resistant transformants were selected after cultured at 28°C for 4 days.

Quantitative PCR

Genomic DNA of *A. chrysogenum* was isolated by the phenol–chloroform method [2]. Absolute quantification was performed to quantify the *cefF* copies of transformant using Mastercycler[®] ep *realplex* equipment (Eppendorf, Germany) and SYBR[®] *Premix Ex Taq*[™] II PCR kit (Takara, Dalian). Standard curve of absolute quantification was created by seven orders of magnitude dilution series (10^{-1} to 10^{-7} dilution) in triplicate of the water solution of pAg1-H3-*cefF*. Samples were analyzed in triplicate from 1/100–1/200 dilution of the original PCR product. Primers 5'-ACGTCGAGCGTCTTCTTCCT-3' and 5'-TTCTTCGCGTGCATGGTG-3' were used in this experiment. Then, 2 μ l of diluted template DNA, 9.5 μ l of water, and 0.5 μ l each of the primers were added to 12.5 μ l of PCR mix and subjected to 40 cycles of PCR (5 s at 95°C and 30 s at 65°C). Melting curve analysis after the cycling confirmed the absence of non-specific products in the reaction. The fluorescence threshold (Ct) values were calculated for standards and samples by using the *realplex* software.

RNA isolation and RT-PCR

Total RNA was isolated from *A. chrysogenum* grown in defined fermentation medium (MDFA) as described previously [21]. RNA was purified by TRIzol and treated with DNase I (Promega) before random 6 mers primed reverse transcription with PrimeScript® RT reagent kit as described by the vendor of the enzyme (Takara, Dalian). No remaining DNA was detected by the PCR amplification of the *cefF* gene. cDNA product of the reverse transcription was used as the template to amplify the *cefF* gene under the same conditions as described in the construction of pAg1-H3-*cefF*.

LC-MS analysis

To analyze the CPC production, *A. chrysogenum* was inoculated in seed medium and defined fermentation

medium (MDFA). All fermentation experiments were performed in triplicate. The CPC production in fermentation broth was measured by bioassay against *M. luteus*. After the fermentation broth was filtered by a 0.2-µm micro PES membrane (MEMBRANA, Germany), 20 µl of sample was directly applied to Agilent 1200 HPLC with Thermo Hypersil Gold C18 column and 6520 Q-TOF mass system for CPC and DAOC production analysis. Standard sample of cephalosporin C zinc salt (cat #3270, Sigma-Aldrich) was used to set up the method of HPLC. The mobile phase was acetonitrile and distilled water (containing 0.05% formic acid) with the gradient from 2:98 (v/v) to 20:80 (v/v) in 20 min at the temperature of 35°C. The flow rate was 1 ml/min. The high-resolution MS analysis was carried out with the electrospray ionization source in the positive mode. The gas temperature and fragmentor were set at 300°C and 115 V, respectively. The drying gas flow was 10 l/min.

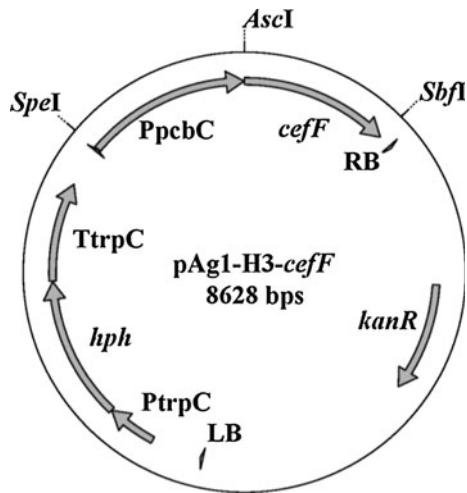


Fig. 1 Map of pAg1-H3-*cefF* carrying the *cefF* gene. *cefF*, DAOC hydroxylase gene under the control of the promoter of *pcbC* (PpcbC) for expression in *A. chrysogenum*; *kanR*, kanamycin resistance gene; *hph*, hygromycin resistance gene under the control of the promoter (PtrpC) and the terminator (TtrpC) of *trpC* gene. *RB* T-DNA right border, *LB* T-DNA left border. Restriction enzymes in **bold** are unique cutting sites in the plasmid

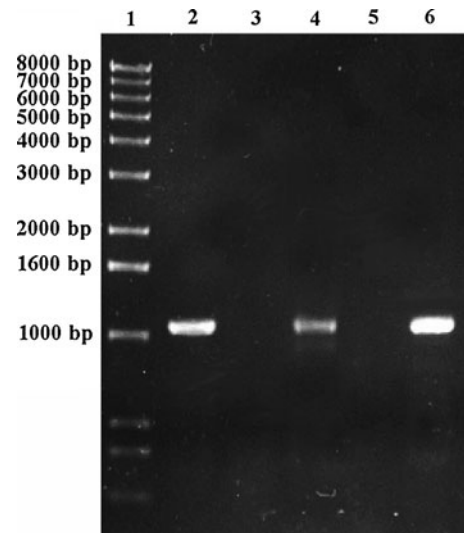


Fig. 2 Transcriptional analysis of *cefF* in *A. chrysogenum*. *Lane 1*, 1-kb DNA marker; *Lane 2*, PCR product using genomic DNA of transformant B6 as a template; *Lane 3*, PCR product using genomic DNA of wild-type strain as a template; *Lane 4*, PCR product using cDNA of transformant B6 as a template; *Lane 5*, PCR product using cDNA of wild-type as a template; *Lane 6*, PCR product using pAg1-H3-*cefF* as positive control

Table 1 Absolute quantification of *cefF* gene copy number

Sample applied	Quantity applied	Amount mean SYBR	Copy number
1/100 diluted genomic DNA	2.4×10^6 molecules	2.51×10^6 molecules	1
1/200 diluted genomic DNA	2.3×10^6 molecules	2.07×10^6 molecules	1

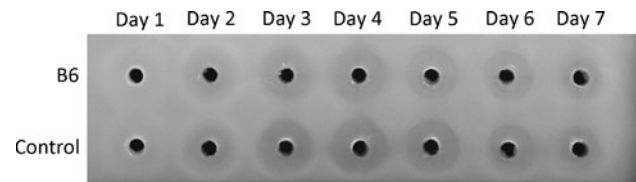


Fig. 3 Bioassay of the fermentation broth from B6 and the wild-type strain against *M. luteus*

Results

Expression of *cefF* in *A. chrysogenum*

To express the *cefF* in *A. chrysogenum*, the plasmid pAg1-H3-*cefF* was constructed. pAg1-H3-*cefF* carried a hygromycin B-resistant cassette (*hph*), kanamycin resistance gene (*kanR*), T-DNA region, and the gene *cefF* under the promoter of *pcbC* (Fig. 1).

The plasmid pAg1-H3-*cefF* was introduced into *A. chrysogenum* through *A. tumefaciens*-mediated transformation. The hygromycin B resistance transformants were selected and confirmed by PCR. These transformants did not show difference in growth and morphological differentiation compared to the wild-type strain (data not shown). For fermentation, one transformant was selected randomly and it was designated as B6. Absolute quantification of *cefF* confirmed that only one copy had been inserted in the chromosome of B6 (Table 1). RT-PCR result showed that *cefF* was transcribed normally in B6, and no signal was observed in the wild-type strain (Fig. 2). However, the CPC production of B6 did not show a significant difference compared to that of the wild-type strain through bioassay against *M. luteus* (Fig. 3).

Analysis of DAOC/CPC in the fermentation broth

The fermentation broth was further analyzed by LC–MS. Due to impurity of the standard sample, DAOC, DAC, and CPC could be detected by comparing the corresponding molecular weight. The HPLC method applied here could separate the DAOC and CPC within an appropriate retention time. The retention time of DAOC was approximately 9.5 min, while the retention time of CPC was 10.2 min (Fig. 4). The DAOC, DAC, and CPC in each sample were analyzed, respectively, by a high-resolution MS monitor. The area of response signal peak was calculated by the Agilent LC–MS software. The resulting value had been processed with statistical analysis (Table 2). According to the peak area, the average ratios of DAOC/CPC and DAOC/DAC in the fermentation broth of the wild-type strain were 14.24 and 7.84, while their average ratios were dramatically decreased to 1.34 and 0.49 in B6 fermentation broth (Table 2). The result also showed that the average ratio of DAC/CPC increased in B6 fermentation broth compared to that of the wild-type strain broth, but not significantly. It is possible that DAC could be converted into CPC efficiently in this strain and keep the dynamic balance between DAC and CPC. The amount of DAOC in the fermentation broth of B6 decreased more than tenfold compared to that of the wild-type strain. This suggested that expression of *cefF* could significantly reduce the content of DAOC in the CPC fermentation broth of *A. chrysogenum*.

Discussion

For 50 years, intensive strain improvement has resulted in a significantly higher titer of the CPC production in *A. chrysogenum* through traditional mutagenesis and genetic engineering. For example, it has been proven that introducing *cefEF* and *cefG*, which was identified as a coding gene of rate-limiting DAC-acetyltransferase, increased the yield of CPC by 40% [23, 29]. Expression of *cefG* under the heterologous *gdh* or *gpd* promoter increased the CPC titer up to two or threefold [14]. Besides amplification of the key biosynthetic enzymes, improvement of corresponding enzymatic activity of precursor synthesis and oxygen supply also stimulated CPC production [9, 21]. However, none of these methods have gained as much achievement as multiple rounds of mutagenesis and screening. Modifying the industrial strain by enhancing the purity of CPC production will be more practical to the modern pharmaceutical industry. In the application reported here, adding a single copy of *cefF* could significantly reduce the ratio of DAOC/CPC. We suggest that this byproduct (DAOC) could be reduced further by switching other promoters or introducing extra copies.

As there is a lack of available genetic tools, molecular engineering in *A. chrysogenum* is still very limited so far. Since the demonstration of successful T-DNA transfer into *Saccharomyces cerevisiae* by *A. tumefaciens*, ATMT has been successfully applied to transform a diverse array of fungi [4, 5, 8, 18]. This technique exhibits many advantages for the genetic analysis of fungi. Our study showed that *A. tumefaciens*-mediated transformation was an efficient method for the introduction of heterologous genes into *A. chrysogenum*. With the high transformation efficiency resulting in one transformant per 1×10^4 spores, more than 30 transformants were collected once in this work, and the genetic screen marker remained stable after five generations.

The high-yielding industrial strains of *A. chrysogenum* were obtained via constant mutagenesis, and a variety of genetic changes occurred and accumulated in these strains, therefore the industrial strains are different from their parents. In contrast to the penicillin-producing strain *Penicillium chrysogenum*, either the wild-type strain or the industrial strains of *A. chrysogenum* contain only one copy of the CPC biosynthetic gene cluster [26]. Though the strain of *A. chrysogenum* examined in this study is the wild-type strain and produced less CPC in the assay of flask fermentation than the industrial strains that had been reported in other references [6, 21, 27], they all produce CPC through the same biosynthetic pathway. We suggest that this method could also be applied in the industrial strain. As presented, the transformant in this work showed a tenfold decline of the accumulation of DAOC. This application should help to decrease the cost of CPC production.

Fig. 4 LC-MS spectrum of the fermentation broths from transformant B6 (a) and the wild-type strain (b)

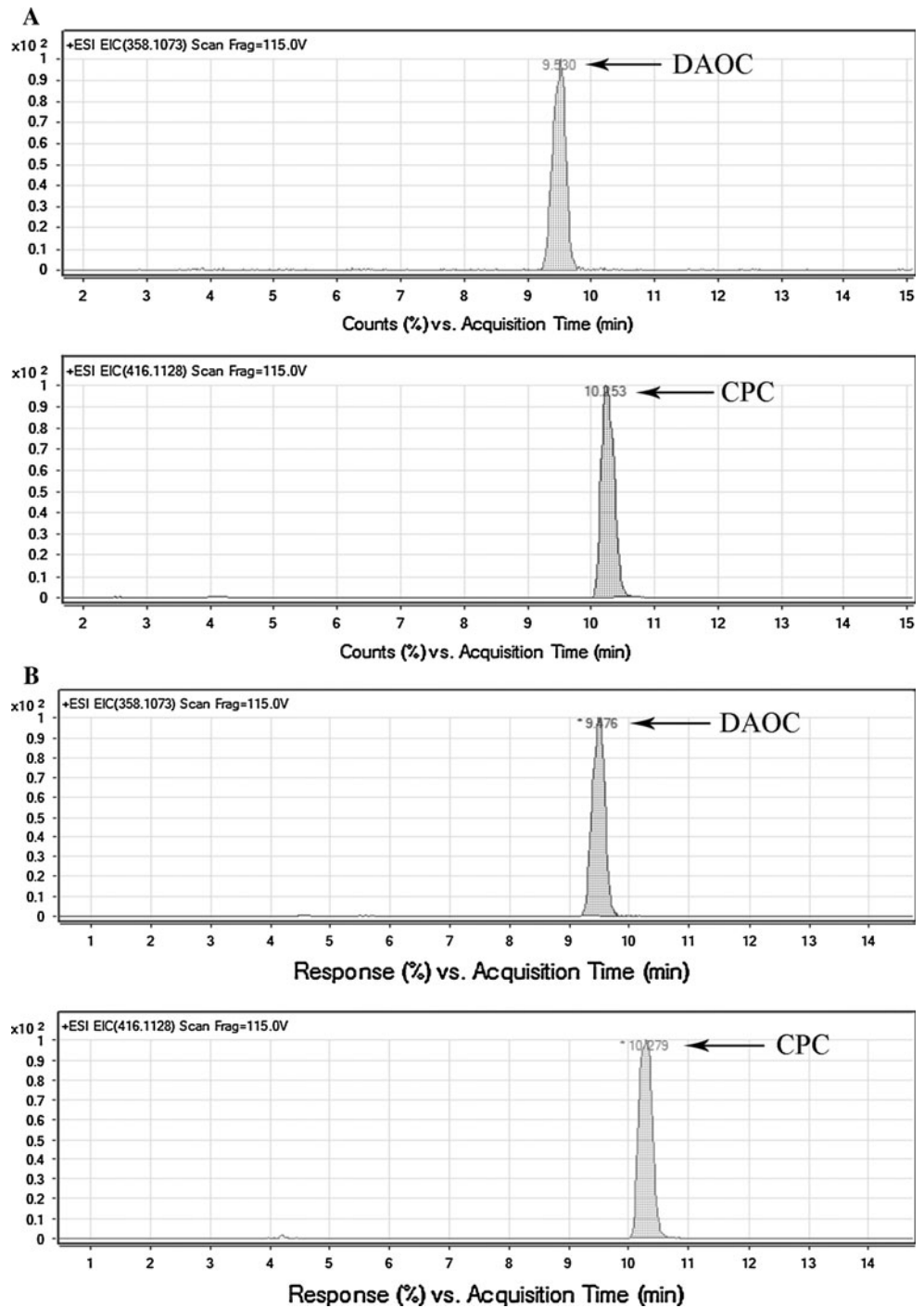


Table 2 Analysis of the DAOC/CPC ratio in the fermentation broth of B6 and the wild-type strain based on the peak areas observed from LC-MS

	Ratio of DAOC/CPC		Ratio of DAOC/DAC		Ratio of DAC/CPC	
	Control	B6	Control	B6	Control	B6
Mean value	14.24	1.34	7.84	0.49	2.20	2.82
Variance	53.77	0.06	23.27	0.01	1.72	0.91
Observations	3	3	3	3	3	3

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