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The Construction of pDH25-pcpC-Vgb as a Recombinant DNA System for the Intracellular Expression of Vitreoscilla Hemoglobin in *Cephalosporium Acremonium*

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Abstract

A recombinant DNA system for the intracellular expression of a bacterial heme-binding protein (*Vitreoscilla hemoglobin*, Vgb) was constructed and named as pDH25-pcpC-Vgb. It could be introduced into a cephalosporin C-producing strain of *Cephalosporium acremonium*. The Vgb-expressing transformants will provide higher internal oxygen concentrations, which will cause higher yields of cephalosporin C.

Keywords: *Cephalosporium acremonium*, *Vitreoscilla hemoglobin*(Vgb), pDH25, Recombinant DNA

1. Introduction

Cephalosporium acremonium is used for industrial production of the β-lactam antibiotic cephalosporin C. For decades, cephalosporin C yields have increased through development of better production strains by classical mutagenesis procedures and optimization of the growth conditions. Recently recombinant DNA technology has been used to overexpress the potentially rate-limiting enzyme deacetoxycephalosporin C synthetase which resulted in increased cephalosporin C production in pilot scale fermentation (Skaturd, 1989, p.477-485). Thus, engineering of filamentous fungi through genetic manipulation represents a promising technology for rational development of improved strains.

Cephalosporin C biosynthesis is regulated by environmental factors such as the phosphate (Zhang, J., 1998, p.242-247), carbon (Martin, J. F., 1982, p.258-268) and oxygen (Hilgendorf, P., 1987, p.247-251) contents of the medium. The overall rate of cephalosporin C synthesis is severely reduced under conditions of low oxygen. Reduction of oxygen supply leads to accumulations of penicillin N, a precursor of cephalosporin C. The mechanism of oxygen control over cephalosporin C biosynthesis is not well understood. Possibly, low oxygen levels directly affect the biosynthesis pathway of cephalosporin C, which includes three oxidation reactions. It is also possible that a more efficient overall metabolism provided by higher oxygen levels indirectly results in higher cephalosporin C yields. Regardless of the mechanism, technology that improve aerobic metabolism in these organisms should have a positive effect on cephalosporin C production (Kallio, P. T., 1994, p.201-218).

Intracellular expression of a bacterial heme-binding protein (*Vitreoscilla hemoglobin*, Vgb) has resulted in higher productively of industrial cell types. For example, the expression of Vgb in the filamentous bacterium *streptromyces coelicolor* resulted in tenfold higher yield of the polyketide actinorhodin in bench scale batch fermentation run under reduced aeration (Magnnolo, S. K., 1991, p.473-476). Also, the efficiency of cloned protein synthesis by oxygen-limited *Escherichia coli* was increased in Vgb-expressing strains (Cullen, D., 1987, p.21-26). Possible mechanisms for Vgb action include increasing the flux of oxygen to the respiratory apparatus, providing higher internal oxygen concentrations, altering the internal redox state, or functioning as an efficient terminal oxidase.

The present work describes the construction of a recombinant DNA system for the intracellular expression of Vgb in filamentous fungi. The recombinant DNA system was named as pDH25-pcpC-Vgb.

2. Experiment

Fungal strain *Cephalosporium acremonium* AS 3.3502 was obtained from The Committee on Type Culture Collection of Chinese Academy of Sciences. Plasmid pDH25 was donated by Pro. Cullen, D. from University of Wisconsin-Madison (Figure 1.). pDH25 provide the structure and hygromycin B resistance gene of recombinant plasmid. Plasmid pVgb and pMW I was obtained from China Agricultural University. The pVgb provides Vitreoscilla hemoglobin gene (Vgb) and the pMW I provides the promoter of isopenicillin N synthetase gene (pcpC) from *Penicillium chrysogenum*. *Escherchia coli* strains Top10 and DH5α were used as recipient hosts for all DNA work. Enzyme and Chemical medicines used in the experiment was purchased from commercial sources, such as Takara Co., Ltd. LB plates with resistance were used for selection. All DNA manipulations were performed by standard protocol (Sambrook, J., 1989).

3. Methods and Results

3.1 Ligation of promoter pcpC and Vgb gene by over-lap PCR

The over-lap PCR premiers was designed as follow: pcpC-OL-Xba I primer1: 5'-GCTCTAGAGGGGCGTCGAGTTGC-3', pcpC-OL primer2: 5'-TTGCTGGTCTAACATGGTGACGGTTTGTCC-3', Vgb-OL primer1: 5'-GGACAAACCGTCACCATGTTAGACCAGCAA-3', Vgb-OL-Xba I primer2: 5'-GCTCTAGATTATTCAACCGCTTGAGCG-3', The sequences of pcpC and Vgb were joined together by the overlapped 15 bp in primer and introduced the Xba I site at each end, thus pcpC-Vgb was constructed. Over-lap PCR was performed by two steps. Firstly, pcpC-OL-Xba I primer1and pcpC-OL primer2 was used for amplifying pcpC, and Vgb-OL primer1 and Vgb-OL-Xba I primer2 for Vgb gene. The amplifying program was showed in Table 1., Table 2. and Figure 2. Secondly, the above product was amplified as template. Table 3. and Figure 2. showed the amplifying program. After amplification and purification, pcpC-Vgb was acquired with Xba I site at each end. The right fragment pcpC-Vgb was confirmed by restriction enzyme digestion and sequencing (Figure 3.).

3.2 Construction of plasmid pDH25-pcpC-Vgb for intracellular expression of Vgb

Plasmid PDH25-pcpC-Vgb was constructed to intracellular express of Vgb in *Cephalosporium acremonium*. Figure 4. graphically illustrates the construction of this plasmid. Plasmid pDH25 and fragment pcpC-Vgb were digested by restriction enzymes Xba I, then were ligated together and named as pDH25-pcpC-Vgb. Plasmid pDH25 was modified to form plasmid pDH25 by addition a 890bp Xba I restriction fragment pcpC-Vgb, which was constructed above. It contains a promoter of isopenicillin N synthetase (pcpC), which can be identified by *Cephalosporium acremonium* ribosome, and can express Vgb successfully if the plasmid integration successful. Plasmid pDH25 contains hygromycin B resistance gene encoding hygromycin B phosphotransferase, which provide a selectable marker for transformation of *Cephalosporium acremonium*. The ampicillinase gene of pBR322 provides the selectable maker of the construct work in *Escherichia coli*. Hygromycin B resistance gene and Vgb have the same transcriptional termination region (Ttrp C) from the tryptophan C gene of *Aspergillus nidulans* in pDH25-pcpC-Vgb. The right plasmid was identified by digestion with restriction enzyme Mlu I (Figure 5.).

4. Conclusion

(1) Plasmid pDH25-pcpC-Vgb can be transformed to *Cephalosporium acremonium*. The transformation will involve chromosomal integration of the plasmid. If it transformed successful, the plasmid will express Vgb and Hygromycin B resistance gene separately, which use its own promoter. The plasmid has the structure of polycistron. The level of Vgb protein expression will be determined by the strain and the location of integration. Further, the EcoR I-Cla I trpC fragment of pDH25 can be conveniently replaced with other promoters for expression Vgb in other filamentous fungi.

(2) The important role played by bacterial hemoglobin in metabolic activities makes this protein a suitable candidate for providing higher internal oxygen concentrations. According the reference (Cullen, D., 1987, p.21-26), Vgb improves antibiotics productivity under low aeration. For both aeration conditions studied, there was no difference in overall growth between Vgb-expressing cells and control cells.

(3) Oxygen plays a major role in cephalosporin C biosynthesis. The tripeptide cyclase, ring expansion, and hydroxylation of deacetoxycephalosprion C steps are all oxygen-requiring processes (DeModena, J. A.,1993, p.926-929). In addition, oxygen regulation may be more significant in the later steps of the pathway due to the accumulation of penicillin N in oxygen-deprived cultures (Khosla, C., 1990, p.849-853). It is possible that Vgb may directly benefit these reactions through its ability to provide higher levels of intracellular oxygen. In a related study, synthesis of the polyketide antibiotics actinorhodin by *streptomyces coelicolor*, another oxygen-intensive pathway, was improved ten-fold in Vgb-expressing cells grown under oxygen-limited conditions (Herold, T., 1988, p.168-173). Possible roles suggested for other unicellular hemoglobins, include oxygen buffering and electron transfer. To help elucidate the role of Vgb in filamentous fungi, it would be of interest to determine its cellular location. Future studies in *Cephalosporium acremonium* will also examine the effect of Vgb on specific enzyme and intermediates of the cephalosporin C pathway, since, to be maximally useful, Vgb must affect cell metabolism in a manner complementary to mutations that may already exist in high-producing industrial strains.

5. Acknowledgement

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Table 1. Reaction System of pcpC PCR in Over-lap PCR Step 1

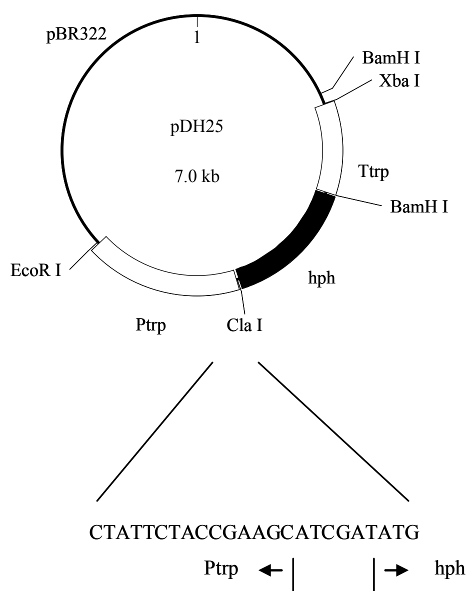
Reactant	Dosage (uL)
Sterile water	13.8
10 *LA Taq Buffer	2
dNTPs (each dNTP is 10 mM)	1
PcpC-OL-Xba I primer1	1
PcpC-OL primer2	1
pMW I	1
LA Taq	0.2
Total	20

Table 2. Reaction System of Vgb PCR in Over-lap PCR Step 1

Reactant	Dosage (μL)
Sterile water	13.8
10 *LA Taq Buffer	2
dNTPs (each dNTP is10 mM)	1
Vgb-OL primer1	1
Vgb-OL-Xba I primer2	1
pVgb	1
LA Taq	0.2
Total	20

Table 3. Reaction System of Over-lap PCR Step 2

Reactant	Dosage (μL)
Sterile water	76.8
10 *LA Taq Buffer	14
dNTPs (each dNTP is10 mM)	7
pcpC-OL-Xba I primer1	7
Vgb-OL-Xba I primer2	7
Vgb(production of PCR above)	7
pcpC(production of PCR above)	7
LA Taq	1.4
Total	140



Ptrp(1250bp): the promoter of *Aspergillus nidulans trpC gene*

hph(1040bp): hygromycin B resistance gene (encoding hygromycin B phosphotransferase)

Ttrp(710bp): the terminator of *Aspergillus nidulans trpC* gene

pBR322(3990bp): BamH I-EcoR I fragment of pBR322 followed by the Xba I-BamH I linker deviced from the PUC18 polylinker

Figure 1. Structure of Vgb Express Vector pDH25

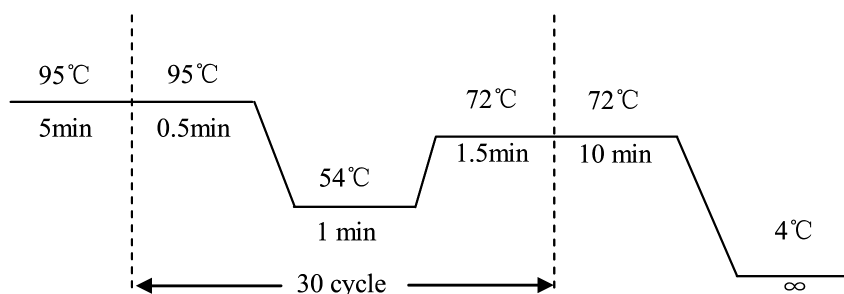


Figure 2. Set of Temperature, Time and Cycle in Over-lap PCR

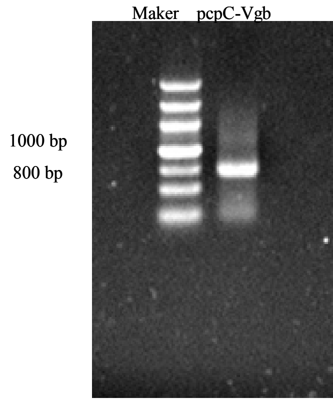


Figure 3. Polymerase Chains Reaction of pcpC and Vgb Over-lap

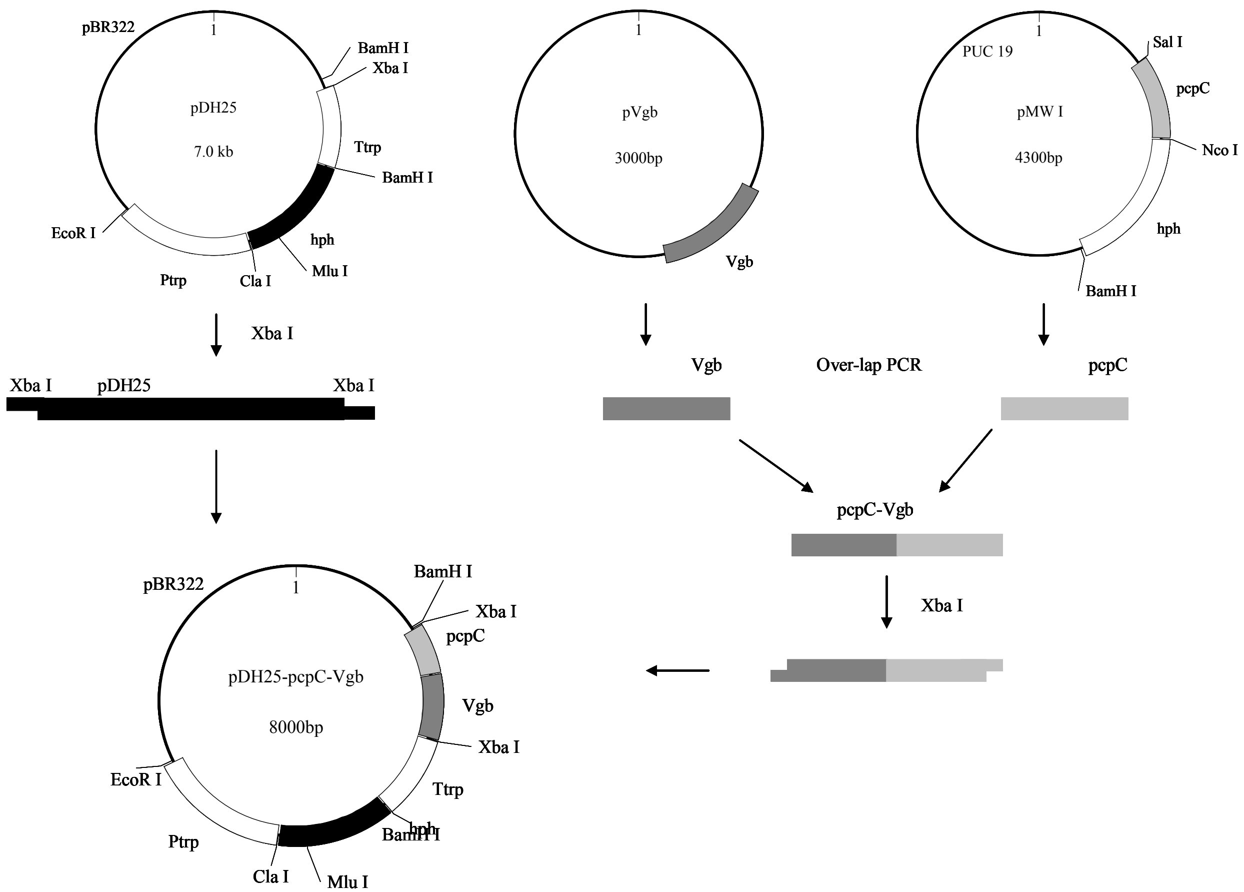


Figure 4. Construction of Plasmid pDH25-pcpC-Vgb.

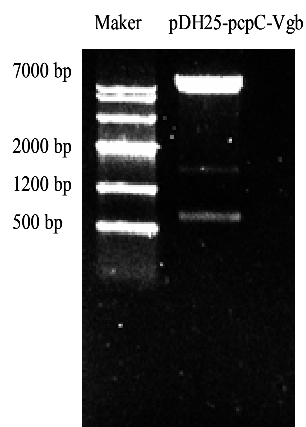


Figure 5. Clave of pDH25-pcpC-Vgb by Mlu I