Metabolic Engineering 13 (2011) 532–543

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

CefR modulates transporters of beta-lactam intermediates preventing the loss of penicillins to the broth and increases cephalosporin production in *Acremonium chrysogenum*

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ARTICLE INFO

Article history:
Received 7 April 2011
Received in revised form
10 June 2011
Accepted 13 June 2011
Available online 21 June 2011

Keywords:
Acremonium chrysogenum
Cephalosporin biosynthesis regulation
Regulatory protein
Gene expression regulation
Cephalosporin transporters

ABSTRACT

The *Acremonium chrysogenum* cephalosporin biosynthetic genes are divided in two different clusters. The central step of the biosynthetic pathway (epimerization of isopenicillin N to penicillin N) occurs in peroxisomes. We found in the "early" cephalosporin cluster a new ORF encoding a regulatory protein (CefR), containing a nuclear targeting signal and a "Fungal_trans" domain. Targeted inactivation of *cefR* delays expression of the *cefEF* gene, increases penicillin N secretion and decreases cephalosporin production. Overexpression of the *cefR* gene decreased (up to 60%) penicillin N secretion, saving precursors and resulting in increased cephalosporin C production. Northern blot analysis revealed that the CefR protein acts as a repressor of the exporter *cefT* and exerts a small stimulatory effect over the expression level of *cefEF* that explains the increased cephalosporin yields observed in transformants overexpressing *cefR*. In summary, we describe for the first time a modulator of beta-lactam intermediate transporters in *A. chrysogenum*.

1. Introduction

Acremonium chrysogenum is an ascomycete fungus, widely used for the industrial production of cephalosporin C (CPC), deacetylcephalosporin C (DAC) and 7-aminodeacetylcephalosporin acid (7-ADCA), compounds that are used to synthesize novel biosynthetic cephalosporins with improved bioactivity (Elander, 2003, Liras and Martín, 2006; Harris et al., 2009). The cephalosporin biosynthetic pathway is well known, since all the implicated genes have been cloned and most of the enzymes have been purified (Dotzlaf and Yeh, 1987; Aharonowitz et al., 1992; Zhang and Demain, 1992; Schofield et al., 1997; Kreisberg-Zakarin et al., 1999; Velasco et al., 1994; Martín, 2000; Lejon et al., 2008). The genes for cephalosporin biosynthesis in *A. chrysogenum* are separated in two clusters that appear to encode two evolutively distinct parts of the pathway (Liras and Martín, 2006). The "early" cluster is located in chromosome VII (Gutiérrez et al., 1999) and includes the biosynthetic genes *pcbAB* (Gutiérrez et al., 1991b), *pcbC* (Samson et al., 1985), *cefD1* and *cefD2* (Ullán et al., 2002b), *cefM* and *cefP* that encode microbody (peroxisome) transporters (Teijeira et al., 2009; Ullán et al., 2010) and *cefT* that encodes a plasma membrane protein (Ullán et al., 2002a). The last three mentioned genes encode proteins involved in the translocation of penicillin N (PenN) and isopenicillin N (IPN) through the peroxisomes and in the secretion of the cephalosporin intermediates DAC, IPN and PenN, respectively. The "late" cluster is located in chromosome I (Gutiérrez et al., 1999) and includes the *cefEF* (Samson et al., 1987) and *cefG* biosynthetic genes, which are transcribed from a single bidirectional promoter (Gutiérrez et al., 1992).

The epimerization of IPN to form PenN is a limiting step during cephalosporin biosynthesis (Ullán et al., 2004), at least partially, due to the fact that the conversion of IPN to PenN takes place inside microbodies (Teijeira et al., 2009; Martín et al., 2010) so importing and exporting proteins that transport these intermediates are required. Indeed, the CefP and CefM proteins are essential for cephalosporin biosynthesis (Teijeira et al., 2009; Ullán et al., 2010). Initially, it was unclear whether those microbodies were authentic peroxisomes but recent cloning of the *cefP* gene and co-localization studies using fluorescent proteins indicate that, indeed, these proteins are located in peroxisomes (Ullán et al., 2010; Martín et al., 2010).

Abbreviations: CPC, cephalosporin C; DAC, deacetylcephalosporin C; IPN, isopenicillin N; PenN, penicillin N; ORF, open reading frame; TDR, transformant disrupted in cefR; TCR, transformant complemented with cefR; TCRP, transformant complemented with cefR and cefP; TSR, transformant overexpressing in cefR

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doi:10.1016/j.ymben.2011.06.004

Modification of the expression of genes encoding limiting steps in cephalosportin biosynthesis is an approach of great interest (Schmitt et al., 2004b; Olano et al., 2008). The regulation of the cephalosporin biosynthetic pathway is still poorly understood. Regulatory mechanisms in *A. chrysogenum* couple extracellular nutritional and environmental inputs to the cephalosporin production rate by complex signal transduction cascades exerted at a transcriptional level (Velasco et al., 1994; Martín and Demain, 2002; Schmitt et al., 2004b; Dreyer et al., 2007) and at a post-transcriptional level (Fierro et al., 2002).

Several proteins implicated in the regulation of cephalosporin biosynthesis at the transcriptional level have been characterized. These proteins may act in a specific manner over the biosynthetic route interacting directly with the genes of the pathway, or in a global manner (if they are involved in developmental changes that affect the pathway). There is scarce knowledge about these proteins and the way they take part in the control of the cephalosporin pathway. Moreover, the number of undiscovered proteins with regulatory functions is probably much higher than the number of those actually known, and their interactions with other proteins (directly related or not with the cephalosporin pathway) are probably much more complex than what is presently known (Schmitt et al., 2004b).

CPCR1 (Schmitt and Kück, 2000; Hoff et al., 2005), AcFKH1 (Schmitt et al., 2004a) and PacC (Jekosch and Kück, 2000; Schmitt et al., 2001) are some of the positive regulators of cephalosporin biosynthetic pathway described until now. To be active, CPCR1 requires to form a complex with AcFKH1 (Hoff et al., 2005). Active CPCR1 regulates the transcription level of *pcbC* gene, hyphal fragmentation and arthrospores formation. Thus, CPCR1 and AcFKH1 represent one example of the interconnection between developmental processes and cephalosporin biosynthesis in *A. chrysogenum* (Schmitt and Kück, 2000; Hoff et al., 2005). Cre1 is a negative regulator of cephalosporin biosynthesis (Jekosch and Kück, 2000). AcVeA is a unique global regulator of cephalosporin biosynthesis and it reduces the expression level of all the biosynthetic genes (Dreyer et al., 2007). Although some secondary metabolite gene clusters contain their own regulators (e.g. the aflatoxin cluster in *Aspergillus*), none of the previous global regulatory genes is linked to the cephalosporin gene clusters.

The available information is even more limited in the case of the cephalosporin secretion genes. It was initially thought that the whole biosynthetic pathway occurs in the cytoplasm (van de Kamp et al., 1999; Evers et al., 2004), but recent evidences show that, at least, the conversion of IPN into PenN, occurs inside peroxisomes (Teijeira et al., 2009; Ullán et al., 2010; Martín et al., 2010). The cephalosporin biosynthetic pathway, requires, therefore, transport of the biosynthetic intermediates in and out of the peroxisomes. Three genes *cefM*, *cefP* and *cefT* have been identified in the early cluster of cephalosporin biosynthesis (Ullán et al., 2002a; Teijeira et al., 2009; Ullán et al., 2010), which are related to the transport of intermediates of cephalosporin biosynthesis. CefP is involved in the import of IPN into the peroxisomes (Ullán et al., 2010), and CefM appears to take part in the transport of PenN from peroxisomes to cytoplasm (Teijeira et al., 2009), while CefT exports hydrophilic penicillins and DAC outside the cell (Ullán et al., 2008b, Nijland et al., 2008). Transporters CefT, CefM and CefP are likely to be under control of a regulation system as occurs with some transporter encoding genes in other eukaryotes and in antibiotic producing *Streptomyces* species (Willems et al., 2008; Le et al., 2009). Therefore, we searched for genes coding regulatory proteins in the cephalosporin C gene clusters. We describe in this article for the first time, a regulatory gene, *cefR*, located in the "early" cluster that controls the transport of intermediates and the secretion of these compounds in *A. chrysogenum*.

2. Materials and methods

2.1. Microorganism and culture media

A. chrysogenum C10 (ATCC 48272) an improved cephalosporin-producing strain (PanLabs, Taiwan) was used as the parental strain in this study. This strain accumulates large amounts of extracellular PenN in addition to cephalosporin C (Ullán et al., 2008a). For sporulation *A. chrysogenum* C10 and its derived mutant strains (indicated in Supplementary Table 1) were grown in solid LPE medium (Ullán et al., 2002b, Ullán et al., 2002a) for 7 days at 28°C. Spores and mycelium collected from six plates of LPE medium were resuspended in 100 ml of seed medium (Ullán et al., 2002b) in 500 ml shake-flasks and incubated at 25°C for 48 h in an orbital incubator at 250 rpm. Ten ml of this seed culture was used to inoculate 100 ml of Shen's defined production (DP) medium. The cultures were incubated in triple-baffled flasks (500 ml; Bellco) containing 100 ml of medium at 25 °C in a rotary shaker (250 rpm). Samples were taken every 24 h, and cephalosporin production was determined by bioassays against *Escherichia coli* ESS2231 as the test strain in plates with penicillinase (from *Bacillus cereus* UL1) to avoid the interference of penicillins as described previously (Gutiérrez et al., 1997).

2.2. Library screening protocol

A gene library of *A. chrysogenum*'s DNA was used for searching new genes. This library was constructed in the *ble*-EMBL3 vector, an EMBL3 derived phage that carries the bleomycin-phleomycin resistance gene (*ble*) of *Streptoalloteichus hindustanus* (Gutiérrez et al., 1991b). Screening of the library was done according to the method described previously by Ullán et al. (2002a).

2.3. DNA isolation and Southern blotting

Genomic DNA of *A. chrysogenum*'s strains were isolated as described previously (Ullán et al., 2002b). Samples of genomic DNAs (3 μg) from *A. chrysogenum* C10 and its transformants were digested with restriction enzymes and separated onto 0.7% agarose gels. The gels were blotted onto Hybond-NX membranes (Amersham-Biosciences) as described by Ullán et al. (2002a). For Southern blot analysis the Dig Easy Hyb system (Roche Diagnostic Corporation) was used. Hybridizations were performed according to the manufacturer's protocol and the hybridization signals were visualized with chemiluminescence and recorded on X-ray film with an exposure time of 5 min.

2.4. Plasmids containing the cefR gene

Plasmids and oligonucleotides used in this study are indicated in Supplementary Tables 2 and 3, respectively. The following plasmids were constructed in this work:

pB5.5Ra and pB5.5Rb. A *Not*I fragment of 5.3 kb containing the *cefR* gene under the control of its own promoter was cloned into the *Not*I site of pBluescript SK $^{+}$II plasmid (Stratagene) in each of the two orientations.

pDR. This plasmid contains the *cefR* gene inactivated. For this purpose, a *Xho*I-*Cla*I fragment of 609 bp of the *cefR* gene was deleted and was replaced with the bleomycin resistance (*ble*) cassette (1.47 kb fragment) from pJL43 (Gutiérrez et al., 1997) into the *Xho*I-*Cla*I sites. The hygromycin resistance (*hph*) cassette, subcloned from pAN7.1 (Punt et al., 1987), was inserted into the *Bam*HI site.

pCR. This plasmid contains a 7.5 kb DNA fragment carrying the *cefR* gene and was used for *trans*-complementation of the *cefR*