Biochimie 115 (2015) 162–176

Contents lists available at ScienceDirect

Biochimie

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

Research paper

PcFKH1, a novel regulatory factor from the forkhead family, controls the biosynthesis of penicillin in *Penicillium chrysogenum*

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ARTICLE INFO

Article history:
Received 9 March 2015
Accepted 18 May 2015
Available online 4 June 2015

Keywords:
Penicillin biosynthesis
Forkhead
Winged-helix
Penicillium chrysogenum
Transcriptional regulation

ABSTRACT

Penicillin biosynthesis in *Penicillium chrysogenum* (re-identified as *Penicillium rubens*) is a good example of a biological process subjected to complex global regulatory networks and serves as a model to study fungal secondary metabolism. The winged-helix family of transcription factors recently described, which includes the forkhead type of proteins, is a key type of regulatory proteins involved in this process. In yeasts and humans, forkhead transcription factors are involved in different processes (cell cycle regulation, cell death control, pre-mRNA processing and morphogenesis); one member of this family of proteins has been identified in the *P. chrysogenum* genome (Pc18g00430). In this work, we have characterized this novel transcription factor (named PcFKH1) by generating knock-down mutants and overexpression strains. Results clearly indicate that PcFKH1 positively controls antibiotic biosynthesis through the specific interaction with the promoter region of the *penDE* gene, thus regulating *penDE* mRNA levels. PcFKH1 also binds to the *pcbC* promoter, but with low affinity. In addition, it also controls other ancillary genes of the penicillin biosynthetic process, such as *phlA* (encoding phenylacetyl CoA ligase) and *ppt* (encoding phosphopantetheinyl transferase). PcFKH1 also plays a role in conidiation and spore pigmentation, but it does not seem to be involved in hyphal morphology or cell division in the improved laboratory reference strain Wisconsin 54-1255. A genome-wide analysis of processes putatively coregulated by PcFKH1 and PcRFX1 (another winged-helix transcription factor) in *P. chrysogenum* provided evidence of the global effect of these transcription factors in *P. chrysogenum* metabolism.

1. Introduction

Penicillium chrysogenum is a filamentous fungus well known by its use in the industrial production of the beta-lactam antibiotic penicillin, which dramatically transformed the global health since Sir Alexander Fleming's discovery of the *Penicillium* that produces this antibiotic [1]. Recent studies have confirmed that the Fleming's original penicillin producing strain and the full genome sequenced strain of *P. chrysogenum* corresponds actually to *Penicillium rubens* [2].

Since the isolation of the original natural fungal isolate, selection and mutagenesis applied during industrial strain improvement programs have raised the penicillin production titers by three orders of magnitude [3]. Many of the modifications responsible for the increased productivity in high-producing strains have been characterized, such as the amplification of the region including the penicillin biosynthetic genes [4], the increase in the number of peroxisomes [5,6], the overexpression of those genes encoding enzymes responsible for the biosynthesis of the amino acid precursors as well as those gene encoding peroxisomal proteins [5]. The high producing strains show a complex rebalancing of global metabolism, including redox reactions, production of energy, biosynthesis of amino acid precursors, virulence or secondary metabolism [7].

Regulatory aspects of penicillin biosynthesis have been also subject of interest for researchers, especially since no penicillin pathway-specific regulators have been found in the region that

Abbreviations: ACV, δ(L-α-aminoadipyl)-L-cysteinyl-D-valine; IAT, IPN acyltransferase; IPN, isopenicillin N; PPTase, 4′-phosphopantetheinyl transferase.

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http://dx.doi.org/10.1016/j.biochi.2015.05.015

contains the penicillin gene cluster (so-called cluster-situated regulators (CSR)) [8,9]. This cluster comprises three genes (*pcbAB*, *pcbC* and *penDE*) encoding the biosynthetic enzymes, which have been largely described and characterized from the molecular and biochemical point of views (for a recent review see Ref. [10]. Briefly, the *pcbAB* gene encodes the multienzyme δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase, which condensates by a non-ribosomal mechanism L-α-aminoadipic acid, L-cysteine and L-valine, thus giving rise to the tripeptide ACV. In the second step of the biosynthetic pathway, the product of the *pcbC* gene, named Isopenicillin N (IPN) synthase (cyclase), catalyses the cyclization of ACV to form IPN, which is the first bioactive compound of the pathway [11,12]. In the last step of the pathway, the IPN acyltransferase (IAT, encoded by the *penDE* gene) substitutes the L-α-aminoadipyl side chain of IPN by activated aromatic acyl side chains, thus forming hydrophobic penicillins. In addition to the *pcbAB*, *pcbC* and *penDE* genes, three ancillary genes are also required for penicillin biosynthesis. The *ppt* gene encodes the 4′-phosphopantetheinyl transferase (PPTase), which activates the ACV synthetase through the addition of a 4′-phosphopantetheine moiety derived from CoA [13]. Another ancillary gene (more precisely a two-component system comprising *trxA* and *trxB*) encodes the NADPH-dependent thioredoxine disulfide reductase (TrxAB), which catalyses the reduction of the oxidized bis-ACV into monomeric ACV so that it can be cyclized by IPN synthase [14]. Finally, acyl-CoA ligases (the main one encoded by *phlA*, formerly named *phl*), are required for the activation of the precursor acyl molecules as CoA thioesters so that they can be incorporated by the IAT during the biosynthesis of hydrophobic penicillins [15]. It is also well-established that penicillin biosynthesis occurs in the cytosol (the first two steps) and peroxisomes, where the acyl-CoA ligases and the IAT are located (reviewed in [6,16]).

In the absence of pathway-specific regulators, penicillin biosynthesis seems to be controlled by several transcription factors in response to environmental and nutritional conditions. It has been known since long that the biosynthesis of this beta-lactam antibiotic is subjected to carbon catabolite regulation [17]. However, it was not until very recently when CreA (the main wide domain regulator responsible for carbon repression in filamentous fungi) was confirmed to participate in carbon repression of penicillin biosynthesis and expression of the *pcbAB* gene [18]. The external pH controls penicillin biosynthesis by means of the transcription factor PacC [19], whereas nitrogen regulation of the penicillin biosynthetic genes is likely mediated by the NRE transcription factor [20,21].

Penicillin biosynthesis is also controlled by global regulators. LaeA is a nuclear protein with a methyltransferase domain that regulates the expression of the penicillin genes and other secondary metabolites in *P. chrysogenum* [22]. LaeA is one of the major components of the velvet-like complex, which comprises at least ten different proteins [23]. Four members of this complex have been characterized so far in *P. chrysogenum*. Velvet A was reported to play an essential role in the biosynthesis of penicillin and in different developmental processes together with LaeA [24]. More recently, other subunits of the velvet complex were identified, namely PcVelB, PcVelC and PcVosA [25]. PcVelC is a strong activator of penicillin biosynthesis and acts in *P. chrysogenum* together with PcLaeA and PcVelA controlling the production of secondary metabolites. In contrast, PcVelB represses penicillin biosynthesis. In addition, PcVelB and PcVosA promote conidiation, whereas PcVelC has an inhibitory effect [25]. Another global regulator that has been recently characterized in *P. chrysogenum* is PcRFX1 [26]. This transcription factor, which is an ortholog of the regulatory factors CPCR1 (*Acremonium chrysogenum*) and RfxA (*Penicillium marneffei*), controls penicillin biosynthesis through the regulation of *pcbAB*, *pcbC* and *penDE* transcription and seems to be involved in the control of several pathways of primary metabolism [26].

In *A. chrysogenum*, CPCR1 positively regulates cephalosporin C biosynthesis by binding at least two sequences at the *pcbAB-pcbC* intergenic region [27,28] and associates with the C-terminus of AcFKH1, which is a member of the forkhead family of proteins [29]. Within the bidirectional *pcbAB-pcbC* promoter, AcFKH1 recognizes two binding sequences [29], which match the consensus 5′-RYMAAYA-3′ determined from 17 different sequences [30]. This suggests a role for AcFKH1 in the biosynthesis of cephalosporin C. Therefore, it is of great interest to study the ortholog of AcFKH1 in *P. chrysogenum* in order to test whether similar regulatory mechanisms operate in those two beta-lactam producers to gain more information on the regulatory aspects of penicillin biosynthesis.

2. Materials and methods

2.1. Strains, media and culture conditions

P. chrysogenum Wisconsin 54-1255 (reference strain for the genome sequencing project) has been re-identified as *P. rubens* [2], although we will use in this article *P. chrysogenum* for consistency with our previous works. *P. chrysogenum* Wisconsin 54-1255 pyrG- (a uridine auxotroph derived from the Wisconsin 54-1255 strain) was also used in this work. These strains were grown in solid Power sporulation medium [31] for 7 days at 28 °C. Flasks cultures for penicillin production were carried out inoculating fresh spores of *P. chrysogenum* in 100 ml of complex inoculum medium CIM [13] without phenylacetate. After incubation at 25 °C for 20 h in an orbital shaker (250 rpm), aliquots (5%) were inoculated in CP complex penicillin production medium [13] with 0.4% potassium phenylacetate and incubated under the same conditions for up to 72 h. Uridine auxotrophs were grown in the presence of 140 μg/ml uridine.

Escherichia coli DH5α cells were used for plasmid amplification, whereas *E. coli* XL1-Blue cells were used for the expression of recombinant PcFKH1. Bacterial cells were grown in Luria-Bertani medium with ampicillin (100 μg/ml).

2.2. Plasmid constructs

Plasmid pJL43-RNAi-Pc*fkh1*, which was used to generate the Pc*fkh1* knock-down transformants, was constructed as follows: Plasmid pJL43-RNAi [32], which contains the *ble* gene marker (for phleomycin resistance), was digested with *Nco*I. Oligonucleotides FKH2silF and FKH2silR (see Supplementary Table S1) were used to amplify a 449-bp exon fragment from Pc*fkh1*, which was digested with *Nco*I and cloned into pJL43-RNAi, thus yielding pJL43-RNAi-*fkh1*. This plasmid was used to transform the *P. chrysogenum* Wisconsin 54-1255 strain.

Plasmid pIBRC43-Pc*fkh1* was used for the overexpression of the *P. chrysogenum* Pc*fkh1* gene and was constructed as follows: Oligonucleotides FKH2NcoIF and FKH2PvuIIR (see Supplementary Table S1) were used to amplify the 2302-bp Pc*fkh1* gene, which was digested with *Nco*I and *Pvu*II. Then, it was inserted between the strong *Aspergillus awamori gdh* gene promoter and the *Saccharomyces cerevisiae cyc*1 transcriptional terminator into pIBRC43 [33], which was previously digested with *Nco*I-*Stu*I. *P. chrysogenum* Wisconsin 54-1255 PyrG-was cotransformed with this plasmid and with the "helper" plasmid pBG [34], which includes the *P. chrysogenum pyrG* gene.

Plasmid pQE-30-Pc*fkh1* was used for the heterologous expression of PcFKH1 in *E. coli* XL1-Blue and was constructed as