Metabolic Engineering 22 (2014) 89–103

Contents lists available at ScienceDirect

Metabolic Engineering

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

New insights into the isopenicillin N transport in *Penicillium chrysogenum*

M. Fernández-Aguado [b], J.F. Martín [b], R. Rodríguez-Castro [a], C. García-Estrada [a], S.M. Albillos [a,1], F. Teijeira [a], R.V. Ullán [a,*]

[a] Institute of Biotechnology of León (INBIOTEC), Av. Real s/n, 24006 León, Spain
[b] Area of Microbiology, Department of Molecular Biology, University of León, Campus de Vegazana s/n, 24071 León, Spain

ARTICLE INFO

Article history:
Received 25 June 2013
Received in revised form
25 November 2013
Accepted 19 January 2014
Available online 28 January 2014

Keywords:
Penicillium chrysogenum
Penicillin biosynthetic pathway
Major facilitator transporter
Isopenicillin N
Peroxisomal membrane protein

ABSTRACT

In *Penicillium chrysogenum* the beta-lactam biosynthetic pathway is compartmentalized. This fact forces the occurrence of transport processes of penicillin-intermediate molecules across cell membranes. Many aspects around this molecular traffic remain obscure but are supposed to involve transmembrane transporter proteins. In the present work, an in-depth study has been developed on a Major Facilitator-type secondary transporter from *P. chrysogenum* named as PenM. The reduction of *penM* expression level reached by *penM* targeted silencing, leads to a decrease in benzylpenicillin production in silenced transformants, especially in SilM-35. On the contrary, the *penM* overexpression from a high efficiency promoter increases the benzylpenicillin production and the expression of the biosynthetic genes. Moreover, when the silenced strain SilM-35 is cultured under penicillin production conditions with 6-aminopenicillanic acid supplementation, an increase in the benzylpenicillin production proportional to the 6-aminopenicillanic acid availability is observed. By this phenomenon, it can be concluded that due to the *penM* silencing the benzylpenicillin transport remains intact but the peroxisomal isopenicillin N import results affected. As a culminating result, obtained by the expression of the fluorescent recombinant PenM-DsRed protein, it was determined that PenM is naturally located in *P. chrysogenum* peroxisomes. In summary, our experimental results suggest that PenM is involved in penicillin production most likely through the translocation of isopenicillin N from the cytosol to the peroxisomal lumen across *P. chrysogenum* peroxisomal membrane.

1. Introduction

Benzylpenicillin [penicillin G (PenG)] was the first of the beta-lactam antibiotics discovered nearly 8 decades ago. This hit opened the way to the discovery of other important beta-lactams, like cephalosporins and cephamycins (Aharonowitz et al., 1992). The use of these antibiotics in the treatment of bacterial diseases in human and veterinarian medicine generated the need to develop a pharmaceutical industry with the capacity to supply the increasing demand of this kind of compounds. The world market of beta-lactams has become a millionaire business that moved $15 billons in 2003 (Ozcengiz and Demain, 2013).

The industrial interest led to the application of different strategies, such as the optimization of the fermentation conditions and genetic modification of the producer strains, by random mutagenesis first and by genetic engineering later (involving beta-lactam related genes) in order to improve the beta-lactam production. As the result of years of research, a deep knowledge has been accumulated about a great variety of different aspects, highlighting the beta-lactam biosynthesis in different producer strains related to genetics, molecular biology and regulation (Evers et al., 2004; Martín et al., 2012a; Ozcengiz and Demain, 2013). All the accumulated knowledge makes today possible new advances around the beta-lactam biosynthesis following more subtle research lines. For example, production rises are expected through the development of *Penicillium chrysogenum* engineered strains with self-protection strengthener mechanisms against the toxic by-products derived from the penicillin biosynthetic route, which would enlarge the mean-life of penicillin related enzymes (Scheckhuber et al., 2013).

These insights have revealed that the biosynthetic genes are arranged in clusters and encode proteins that exert their enzymatic

Abbreviations: Wis 54-1255, Wisconsin 54-1255; IPN, isopenicillin N; PenG, penicillin G, benzylpenicillin; 6-APA, 6-aminopenicillanic acid; DP medium, defined production medium; CP medium, complex production medium; MFS, major facilitator superfamily, TMS, transmembrane spanning domains; RNAi, RNA interference; IOD, integrated optical density; EGFP, green fluorescent protein; DsRed, red fluorescent protein

* Corresponding author. Fax: +34 987 210388.
E-mail address: rvicu@unileon.es (R.V. Ullán).
[1] Present address: Area of Biochemistry and Molecular Biology, Department of Biotechnology and Food Science and Technology, University of Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Spain.

1096-7176/$ - see front matter © 2014 International Metabolic Engineering Society. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymben.2014.01.004

activities in different subcellular compartments. The compartmentalization characterizes the beta-lactam biosynthetic pathways, having a deep knowledge of the related features for *P. chrysogenum* and *Acremonium chrysogenum*. The starting enzyme of the beta-lactam biosynthetic pathway, the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) resides in the cytoplasm (van der Lende et al., 2002). The ACVS is encoded by the *pcbAB* gene and condenses the amino acid precursors L-cysteine, L-α-aminoadipate and D-valine to form the intermediate δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). The isopenicillin N synthase (IPNS), the second enzyme of the route, encoded by the *pcbC* gene, is also a soluble cytoplasmic protein as confirmed by subcellular fractionation and immunoelectron microscopy (van der Lende et al., 2002). The IPNS cycles the ACV to produce the hydrophilic isopenicillin N (IPN) (Aharonowitz et al., 1992).

At this point of the biosynthetic pathway, the IPN can be directed towards the production of PenG (as occurs in *P. chrysogenum* and *Aspergillus nidulans*), cephalosporin C (as happens in the *P. chrysogenum*'s related fungi *A. chrysogenum*) or cephamycin C (as is the case of the less related prokaryote strains *Streptomyces clavuligerus* and *Amycolatopsis lactamdurans*).

In *P. chrysogenum*, the IPN is used by the peroxisomal enzyme isopenicillin N acyltransferase (IAT), encoded by *penDE* (Müller et al., 1991; García-Estrada et al., 2008). The IAT exchanges the side chain of the IPN by a more hydrophobic one, *i.e.* an activated phenylacetic acid residue added by peroxisomal acyl-CoA ligases (Lamas-Maceiras et al., 2006; Wang et al., 2007; Yu et al., 2011), to form the end-product (PenG) that has to be sorted to the extracellular medium. In *A. chrysogenum*, the genes *cefD*1 and *cefD*2 encode two enzymes that use IPN as substrate to synthesize penicillin N (PenN) (Ullán et al., 2002b, 2004) into the peroxisomal matrix (Teijeira et al., 2009). Later, in the cytosol, PenN is converted into cephalosporin C (van de Kamp et al., 1999; Martín et al., 2010). Lately, in *P. chrysogenum* the expression of heterologous genes from other beta-lactam producers (like *A. chrysogenum* or *S. clavuligerus*) has made possible the enlargement of the biosynthetic capability of the first. Some *P. chrysogenum* engineered strains, for example, produce cephalosporin-precursor molecules. In these new strains of *Penicillium*, the actual aim is the suppression of metabolic pathways competing with the beta-lactam engineered route, thus providing its optimal exploitation towards the formation of the cephem-precursor of interest (Ullán et al., 2007; Harris et al., 2009; Veiga et al., 2012).

The occurrence of the different steps of beta-lactam biosynthesis in different subcellular compartments makes the necessary transport of precursors and intermediates across cellular membranes. These transport processes would be mediated by membrane transporter proteins from any of the superfamilies or families included in the Multidrug Resistance (MDR) protein class (Martin et al., 2005) [the ATP-Binding cassette (ABC) superfamily, the Major Facilitator superfamily (MFS), the Multidrug and Toxic compound extrusion (MATE) family, the Small multidrug resistance (SMR) family or the Resistance Nodulation Division (RND) family], all of them characterized by their ability to transport a wide variety of dissimilar organic compounds. However, despite the great amount of knowledge acquired about the different biosynthetic pathways, yielding beta-lactams as their final bioactive products, the transport processes associated to the compartmentalization that characterizes them, constitute an interesting area that still remains largely unexplored. In fact, only a few transporter proteins involved in beta-lactam biosynthetic pathways have been described to date.

One of these transporters is the putative cephamicin transporter encoded by *cmcT* gene that exists in the cephamicin cluster of the producing bacteria *S. clavuligerus* and *A. lactamdurans*. The protein CmcT is a member of the MFS of secondary transporters, being located in the cytoplasmic membrane and being responsible for the export of cephamicin (Coque et al., 1993; Liras, 1999).

Within the early cephalosporin C biosynthetic cluster of *A. chrysogenum*, a meticulous work has been made with the result of the biochemical and molecular characterization of three genes encoding membrane transport proteins. These three transport proteins are proved to take part somehow in the cephalosporin biosynthesis. The *cef*T gene encodes a MFS transporter involved in the secretion of beta-lactams containing the α-aminoadipic-derived side chain, like IPN, PenN and deacetylcephalosporin C (Ullán et al., 2002b, 2008b). CefP is a peroxisomal membrane transporter involved in the translocation of the intermediate IPN from the cytosol to the peroxisomal lumen (Ullán et al., 2010). The other MFS transporter, the one encoded by *cefM* is also located in the peroxisomal membrane and is in charge of the exportation of PenN to the cytoplasm from the lumen of this organelle. In *P. chrysogenum*, our group described the MFS transporters PenV and PaaT. The first one is a vacuolar membrane protein which plays an important role in the formation of the ACV tripeptide, probably due to its function as an amino acid permease, supplying precursors from the amino acid vacuolar pool (Fernández-Aguado et al., 2013a). The PaaT, showing homology with *A. chrysogenum* CefT, is located in the peroxisomal membrane and is supposed to intervene in the internalization of phenylacetate in the peroxisomes, where it enters in the penicillin biosynthetic pathway as the side chain of the PenG (Fernández-Aguado et al., 2013b). In *P. chrysogenum* CGMCC 3.5129, the gene *penT*, homologue of *cefT* too, is reported to be located in the plasma membrane and related to the sensitivity to phenylacetate (Yang et al., 2012). Also related to the transport of phenylacetate in *P. chrysogenum*, the transporterABC40 has been reported to act as an ATP-dependent extrusion system of some weak acids such as phenylacetate, benzoate and sorbate, protecting cells from the harmful acidification of their cytoplasm. ABC40 encoding gene is reported to be more expressed in low penicillin producing strains of *P. chrysogenum* in line with a less efficient detoxification of phenylacetate *via* its incorporation to the beta-lactam pathway (Weber et al., 2012).

Despite the fact that even though MFS transporters are involved in the cell growth and homeostasis and are present in all the evolution kingdoms in nature, they have not been characterized sufficiently, maybe due to the intrinsic difficulty in working with hydrophobic membrane proteins or in the evaluation of the proper transport process. The present work faces several approaches to continue unraveling the MFS-mediated transport processes linked to the PenG biosynthetic pathway in *P. chrysogenum.* Here the characterization of the *penM* gene of *P. chrysogenum*, which encodes an MFS-like protein proved to be in the peroxisomal membrane, is described. This protein is somehow involved in the penicillin biosynthesis given the clear phenotypic effects on the penicillin production rate provoked by the *penM*-silencing and *penM*-overexpression strategies explored in this work in *P. chrysogenum*-engineered strains.

2. Materials and methods

2.1. Strains, media and culture conditions

In this study, *P. chrysogenum* Wisconsin 54-1255 (ATCC 28089/DSM 1075) was used as the strain of reference, along with a low-penicillin producer strain (800 mg/L in CP medium) containing a single copy of the penicillin gene cluster (Cantoral et al., 1987; Fierro et al., 1995) that derives from the wild type *P. chrysogenum* NRRL 1951. The engineered strains made in this work were obtained by transformation of *P. chrysogenum* Wisconsin 54-1255 (Wis 54-1255) with different integrative vectors.