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COMPARTMENTALIZATION IN CEPHALOSPORIN C BIOSYNTHESIS BY INDUSTRIAL STRAINS OF *ACREMONIUM CHRYSOGENUM*

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Abstract: Cephalosporin C biosynthesis is a compartmentalized process located mainly in the sub-apical, productive, non-growing cells of the hyphae, which under the conditions of the industrial technology build well-dispersed flocculent mycelia. In this paper, the cephalosporin C production by industrial strains of *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*) is described, including the central role of peroxisomes in the biosynthesis and secretion of this antibiotic and other β-lactams. The localization of the pathway of cephalosporin C biosynthesis and important transport steps of intermediates and the end-products are also discussed.

Ultrastrukturalna organizacja komórek grzybni *A. chrysogenum* podczas produkcji cefalosporyny C na skalę przemysłową

Streszczenie: W niniejszej pracy omówiono rolę peroksysomów w wytwarzaniu cefalosporyny C przez przemysłowe szczepy *A. chrysogenum*. Przedstawiono lokalizację enzymów szlaku biosyntezy cefalosporyny C w dojrzałych metabolicznie aktywnych nierosnących komórkach grzybni. Omówiono także niektóre aspekty zwiększania wydajności szczepów przemysłowych.

Key words: *A. chrysogenum*, cephalosporin C, biosynthesis, peroxisomes
Słowa kluczowe: *A. chrysogenum*, cefalosporyna C, biosynteza, peroksysomy

1. Introduction

Cephalosporin C was shown to be active against Gram-positive and Gram-negative bacteria. At present, *A. chrysogenum* is cultured worldwide to yield approximately 2,500 tons (annual production) of semi-synthetic derivatives of cephalosporin C which are mainly used as broad-spectrum antibiotics for the treatment of bacterial infections. This review starts with a summary of the pathway of cephalosporin C biosynthesis, followed by an overview of the compartmentalization in cephalosporin C biosynthesis by *A. chrysogenum*. Then an outline of the role of peroxisomes in cephalosporin C biosynthesis is given, and additionally the industrial strain improvement is detailed.

2. The pathway of cephalosporin C biosynthesis

The pathway of cephalosporin C biosynthesis is shown in Fig. 1 and detailed in Fig. 2. The biosynthesis of this antibiotic starts by the non-ribosomal condensation of three amino acids, i.e.: L-α-aminoadipic acid (non-proteinogenic amino acid – in fungi synthesized by a specific aminoadipate pathway, which leads to the formation of lysine), L-cysteine and L-valine (ubiquitous amino acids) to form the tri-peptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). This reaction is catalyzed by the ACV synthetase (ACVS) – encoded by the *pcbAB* gene [1, 14]. ACVS in sequence activates the three amino acids with ATP to form aminoacyl-adenylates, binds them to the enzyme as thioesters, epimerizes the L-valine to D-valine, links together the three amino acids to form the ACV-peptide, and releases the tri-peptide from the enzyme by the action of an internal thioesterase activity [17]. Subsequently, the ACV tri-peptide is cyclized to isopenicillin N (IPN) by the isopenicillin N synthase (IPNS, cyclase) – encoded by *pcbC* gene. The IPN synthases require Fe^{2+}, molecular oxygen and ascorbate. They remove four hydrogen atoms from the ACV tri-peptide [for review, see 17]. In this oxidative ring closure reaction a bi-cyclic penam nucleus, consisting of a β-lactam and thiazolidine ring is formed. In *Penicillium*, IPN is

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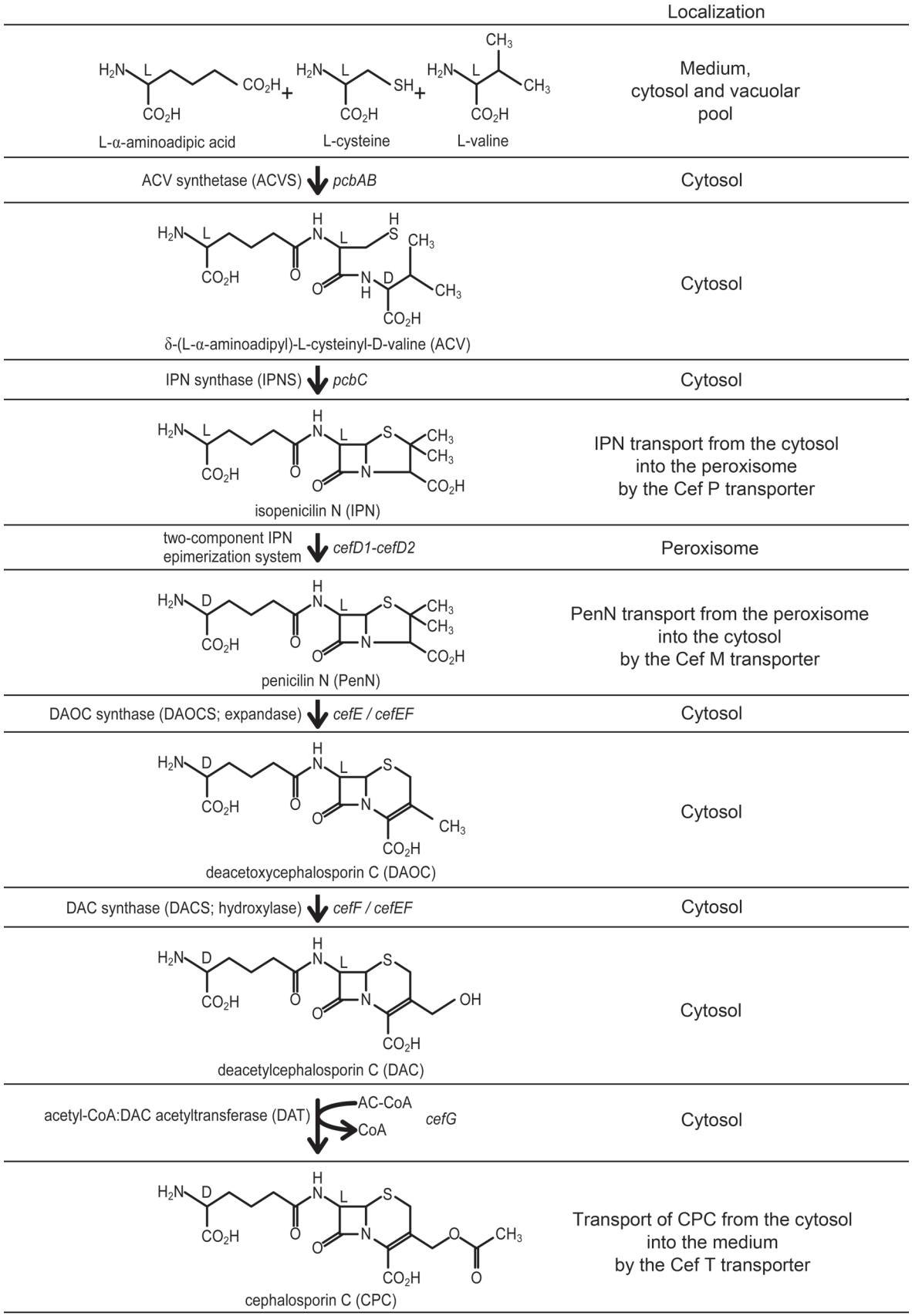

Fig. 1. Compartmentalization of cephalosaporin C biosynthetic pathway in the productive sub-apical non-growing mycelilal cells of *A. chrysogenum.* Note the peroxisomal conversion of isopenicillin N into penicillin N by the two-protein CefD1-CefD2 epimerization system (details in Fig. 2). ACVS, IPNS, DAOCS, DACS and DAC acyltransferase are cytosol enzymes. In *Streptomyces clavuligerus* [27] and other bacteria the enzymes DAOCS and DACS are encoded by the separate *cefE* and *cefF* genes. The proposed localization of the β-lactam transporters, i.e.: CefP, CefM and CefT are indicated (Author: W. Kurzątkowski).

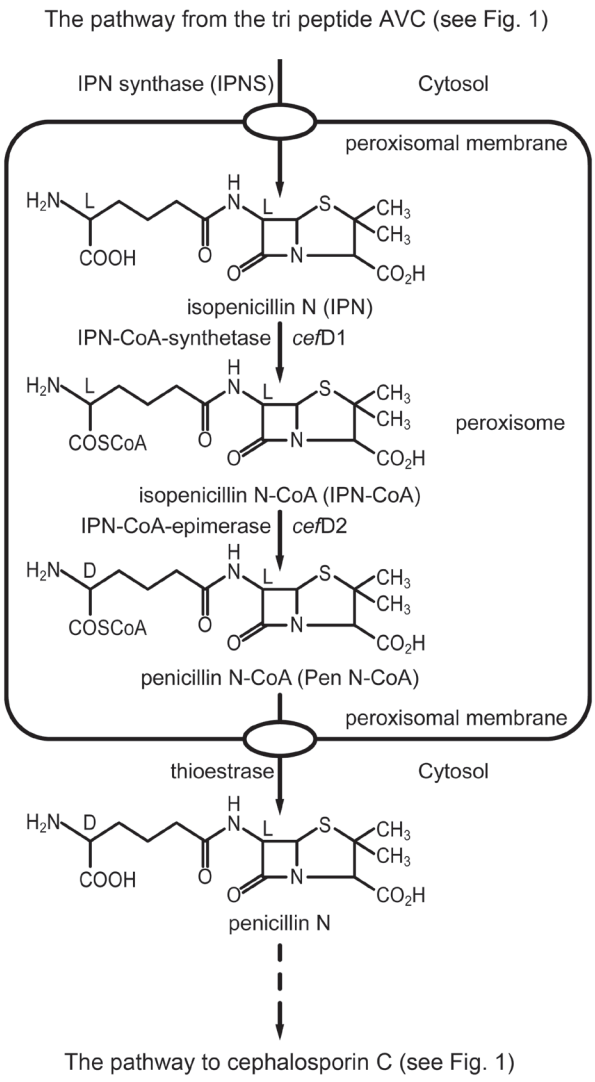

Fig. 2. The peroxisomal conversion of IPN to Pen N by the two protein cefD1-cefD2 epimeryzation system composed of: IPN-CoA synthetase and IPN-CoA epimerase is shown. Al other steps in cephalosporin C biosynthesis are located in the cytosol (Author: W. Kurzątkowski)

converted to hydrophobic penicillins, e.g. penicillin G [15, 16, 35, 36]. The formation of IPN is the branch point of penicillin and cephalosporin biosynthesis. In *Acremonium*, streptomycetes and all other cephalosporin and cephalomycin producers, IPN is converted (Fig. 1 and Fig. 2) to penicillin N (D-isomer of IPN) by an epimerization CefD1-CefD2 system [for review, see 29]. The following step in cephalosporin C biosynthesis is the enzymatic expansion of the five-membered thiazolidine ring of penicillin N to a six-membered dihydrothiazine ring. This conversion is catalyzed by the deacetoxycephalosporin C (DAOC) synthase (DAOCS) – encoded by the *cefEF* gene. The DAOCS requires Fe^{2+}, molecular oxygen and α-ketoglutarate to form DAOC and succinic acid. This enzyme does not recognize IPN, penicillin G or the 6-aminopenicillanic acid as substrates [for review, see 17; Wu et al. 37]. The DAOCS from *A. chrysogenum* is also able to catalyze the hydroxylation of DAOC at C-3 forming deacetylcephalosporin C. In the last step of cephalosporin C biosynthesis, the transfer of an acetyl-moiety from the acetyl-coenzyme A to hydroxyl group on the sulfur-containing ring of deacetylcephalosporin C leads to the formation of cephalosporin C, which possesses high antibacterial activity [29]. This reaction is catalyzed by the acetyl-Coenzyme A (CoA):DAC acetyltransferase – encoded by *cef*G gene.

3. Secondary metabolism of cephalosporin C

Penicillins and cephalosporins, like many other secondary metabolites, have unusual chemical structures [2, 3, 9, 15, 16, 18]. Cephalosporin C produced by the fungi *A. chrysogenum*, *Paecilomyces persicinus*, *Kallichroma tethys* and other deuteromycetes contains the cephem nucleus composed of a six-membered dihydrothiazine ring fused to the β-lactam ring. This β-lactam has a D-α-aminoadipyl side-chain attached to the C-7 amino group [13]. The most important feature classifying these antibiotics as secondary metabolites is the non-ribosomal condensation of the ACV tri-peptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine that is characterized by the unusual δ-α peptide binding between the carboxylic group located in the position δ of the L-α-aionadipic acid and the α-amino group of the L-cysteine. Further features are the LLD-configuration of the ACV-tri-peptide [35, 36] as well as the formation of bi-cyclic penam (penicillins) or cephem (cephalosporins and cephamycins) nuclei [16]. In the primary metabolism the peptides are synthesized in the process of transcription and translation and are composed of linearly arranged amino acids combined with peptide bindings formed from carboxylic groups and amino groups situated in the position α of the amino acids. Other feature is the branching from the pathways of the primary metabolism (L-α-aminoadipic acid, L-cysteine and L-valine) of unusual short one-way biosynthetic roads leading, in filamentous fungi, to the formation of penicillins and cephalosporins that exist in the cells of the producers as non-metabolized end-products which are finally secreted from the cells of the mycelium into the cellular medium.

4. Cellular localization of enzymes involved in cephalosporin C biosynthesis (compartmentalization)

The proposed compartmentalization of the cephalosporin C biosynthetic pathway in the sub-apical non-growing cells of the mycelium is shown in Figs. 1, 2.

The enzymes catalyzing the last two steps in penicillin biosynthesis (phenylacetyl-CoA ligase and IPN acyltransferase) are located in peroxisomes – also called microbodies [5, 15, 16, 18, 22, 34], as shown by immunoelectron microscopy. Similarity (Fig. 2), the *Acremonium* two-protein CefD1-CefD2 epimerization system converting IPN to penicillin N is also located in peroxisomes [17]. Al other enzymes of the pathway of cephalosporin C biosynthesis, i.e.: ACVS, IPNS, DAOCS, DACS, DAC acetyltransferase are soluble cytosol enzymes [33]. This distinct sub-cellular compartmentalization implies intracellular transport of IPN and penicillin G in the penicillin G pathway or IPN and penicillin N in the cephalosporin C route as well as enables the spatial separation of procedures and enzymes simplifying the regulation and optimization of the processes involved.

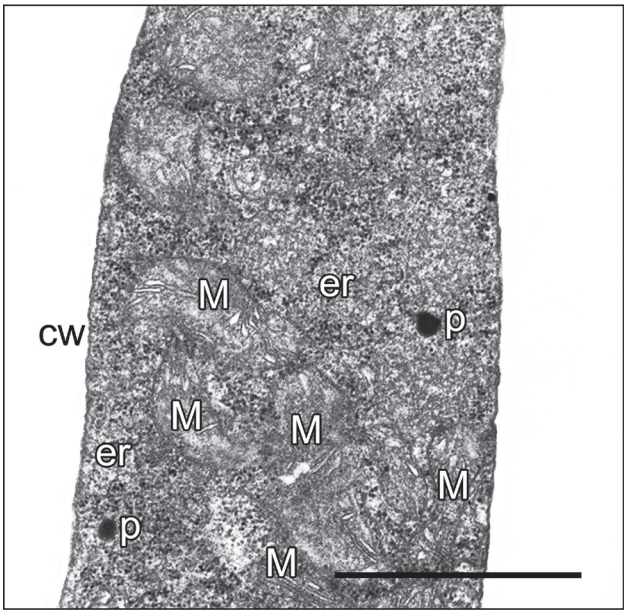

Fig. 3. *A. chrysogenum* (high-yielding, industrial strain). The young-mature sub-apical productive non-growing mycelial cell can be characterized as follow. The protein reach cytoplasm is densely packed with ribosomes and mitochondria (M), that are associated with peroxisomes (p) of about 0.1–0.2 μm in diameter. The cell wall (cw) is composed of one thin electron transparent layer, bar = 1 μm (Author: W. Kurzątkowski).

5. The role of peroxisomes in cephalosporin C biosynthesis

The biosynthetic pathway of cephalosporin C is compartmentalized in the productive mycelial cells of *A. chrysogenum* and takes place in the cytosol, and in the peroxisomes, like the biosynthetic pathway of penicillin G in *Penicillium chrysogenum*. The peroxisomal conversion of IPN to penicillin N by the protein CefD1-cefD2 epimerization system in cephalosporin C biosynthesis [32] is presented in Fig. 2. This compartmentalization implies intracellular transport of IPN and Pen N in the cephalosporin C pathway. The CefM protein seems to act as a transporter between the peroxisomal lumen and the cytosol to provide the penicillin N intermediate for the reactions catalyzed by the expandase/hydroxylase and acetyl-CoA:DAC acetyltransferase. Three transporters, i.e.: CefP, CefM and CefT are considered in the transport of intermediates and/or secretion of cephalosporins [5, 24, 31, 33]. The results from our experimental programs using transmission electron microscopy exhibit in the sub-apical productive non-growing cells of the high-yielding industrial strain of *A. chrysogenum* numerous mainly spherical peroxisomes from 0.1 μm up to 0.4 μm in diameter which are frequently accompanied by numerous mitochondria and polyribosomes (Fig. 3). Such an arrangement of peroxisomes may increase the IPN-CoA synthetase and IPN-CoA-epimerase (CerfD1/CefD2 epimerization system) supplying the efficacy in cephalosporin C biosynthesis. Small peroxisomes could be observed rarely in the apical cells located 1.0–2.0 μm from the apex of the hyphae and in the sub-apical growing regions of the industrial strain. In the late-apical highly vacuolated cells massive autophagy was observed. In these cells autophagosomes-like structures could be observed. Autophagy was accompanied by cytoplasm and peroxisome degradation. Small peroxisomes were also rarely visible in the cells of the low-cephalosporin-producing strain *A. chrysogenum* (data no shown). This results support strongly the participation of peroxisomes in cephalosporin C biosynthesis. Our observations are consistent with the reported positive correlation between penicillin G biosynthesis and the number of large peroxisomes in sub-apical productive non-growing mycelial cells of *P. chrysogenum* [11, 12, 15, 16, 19, 23]. Two models of peroxisome development have been documented, the *de novo* synthesis from the endoplasmic reticulum and multiplication by fission of pre-existing organelles [2, 3, 7, 8, 15, 16, 19, 20, 21, 24, 26, 28, 30].

6. Industrial strain improvement

Classical strain improvement has yield industrial *A. chrysogenum* strains that produce high titers of cephalosporin C. In approximately 50 years, intensive strain improvement programs mainly mutation and selection resulted in production of strains that yield significantly higher titer of the cephalosporin C than wild-type strains. At present, the genetic engineering techniques together with conventional strain improvement procedures seem to be a perspective to increase the yield of cephalosporin C production. The industrial

strains exhibit elevated transcription of genes involved in β-lactams biosynthesis [35, 36]. At present, for industrial strain improvement, further experimental work is required to elucidate the role of transporters in the production of β-lactam antibiotics to increase translocation of the intermediates and secretion of the final products [11].

7. The β-lactams in the treatment of various bacterial infections – mode of action

Cephalosporins are members of the large group of β-lactam antibiotics, which inhibit the growth of Gram-negative and Gram-positive bacteria at low concentration. These antibiotics are widely used in human medicine. All β-lactams contain a four-membered β-lactam ring closed by an amide bound that is immediately involved in the mode of action of these antibiotics [6] by acylation of the hydroxyl group of serine located in the active centre of DD-carboxypeptidase/transpeptidases (DD-peptidases). Such an acylation results in inhibition of DD-peptidases (catalyzing the cross-linking of the peptidoglycan) causing the death of bacterial cells. The mechanisms of the always increasing resistance of bacteria to the natural and semi-synthetic β-lactams include numerous mutations leading to the production of a large number of sensitive β-lactamases (accepting the novel β-lactams) and/or modified DD-peptidases (not accepting the novel β-lactams). Therefore, the necessary prerequisite is the continuous search for novel β-lactams that are resistant to the actually produced β-lactamases and efficient against the DD-peptidases.

8. Conclusions

The sub-apical mature non-growing peroxisomal cells of the high-yielding strain are privileged in cephalosporin C biosynthesis. The *A. chrysogenum* two-protein CefD1-CefD2 epimerization system converting IPN to Penicillin N is located in peroxisomes. Other enzymes of the pathway of cephalosporin C biosynthesis are soluble cytosolic enzymes. For improvement of industrial strains further work is required to elucidate the role of the CefP and CefT transporters in cephalosporin C biosynthesis by *A. chrysogenum*. The inhibition of autophagy is also a possibility for industrial strain improvement.

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