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PENICILLIN G PRODUCTION BY INDUSTRIAL STRAINS OF *PENICILLIUM CHRYSOGENUM*

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Abstract: The role of peroxisomes in penicillin G production by industrial strains of *Penicillium chrysogenum* is discussed. Penicillin G biosynthesis is a compartmentalized process mainly located in sub-apical productive vacuolated non-growing cells of the mycelium. The cellular localization of the pathway of penicillin G biosynthesis is presented, including some putative enzymatic and transport steps of precursors, intermediates, side-products and end-products. A phenylacetic acid detoxification hypothesis of penicillin G biosynthesis is suggested.

Produkcja penicyliny G przez przemysłowe szczepy *Penicillium chrysogenum*

Streszczenie: Omówiono rolę peroksysomów w biosyntezie penicyliny G wytwarzanej przez przemysłowe szczepy *P. chrysogenum*. Proces biosyntezy penicyliny G jest zorganizowany głównie w dojrzałych metabolicznie aktywnych nierosnących komórkach grzybni, w których widoczne są wakuole. Przedstawiono lokalizację procesu biosyntezy penicyliny G w komórkach grzybni, tj. lokalizację enzymów oraz drogi transportu prekursorów, metabolitów pośrednich, produktów ubocznych i produktów końcowych. Zasugerowano hipotezę, że biosynteza penicyliny G jest procesem detoksykacyjnym chroniącym komórki producenta przed dozowanym do brzeczki fermentacyjnej kwasem fenylooctowym (prekursor biosyntezy penicyliny G), który w wysokich stężeniach jest toksyczny dla grzybni.

Key words: biosynthesis, penicillin G, *P. chrysogenum*, peroxisomes
Słowa kluczowe: biosynteza, *P. chrysogenum*, penicylina G, peroksysomy

1. Introduction

Penicillin G and derived β-lactam antibiotics have significantly transformed health care and quality of life in the 80 years since Fleming's discovery of *Penicillium* that produces penicillins [7]. Large-scale penicillin G production is a result of industrial strain improvement, representing numerous mutagenesis and selection [34]. β-Lactams have been used extensively for treatment of various bacterial infections for more than half a century, and today penicillins are commodity-type products with annual production volumes exceeding 60,000 tons. Improvement of the industrial strains producing β-lactams is therefore of great economical importance [32]. Below, we discuss different aspects of penicillin G biosynthesis.

2. The pathway of penicillin G biosynthesis

The pathway of penicillin G biosynthesis is shown in Fig. 1. Penicillin G is synthesized by intracellular condensation of activated precursor amino acids L-α-aminoadipic acid (A), L-cysteine (C) and L-valine (V) to δ-(L-α-aminoadipil)-L-cysteinyl-D-valine (ACV) tri-peptide formed by the ACV synthetase (ACVS) – encoded by the *pcb*AB gene [1, 14]. This tri-peptide is converted to isopenicillin N (IPN) by the action of IPN synthase (IPNS) – encoded by the *pcb*C gene. In this oxidative ring closure reaction the bi-cyclic penam nucleus is formed consisting of β-lactam and thiazolidine rings [36, 37]. In the last step, the IPN acyltransferase (IAT) – encoded by the *pen DE* gene synthesizes penicillin G by substitution of the L-α-aminoadipyl side chain of IPN by the phenylacetyl side chain. The phenylacetic acid (PA) – precursor of penicillin G biosynthesis requires previous activation by specific phenylacetyl-CoA ligase (PCL) – encoded by *phl gene* [2, 37, 38].

3. Secondary metabolism of Penicillin G

Penicillin G is a secondary metabolite of *P. chrysogenum* [4, 5, 11, 19]. The main feature classifying this compound as a secondary metabolite is the non-ribosomal condensation of the tri-peptide ACV characterized

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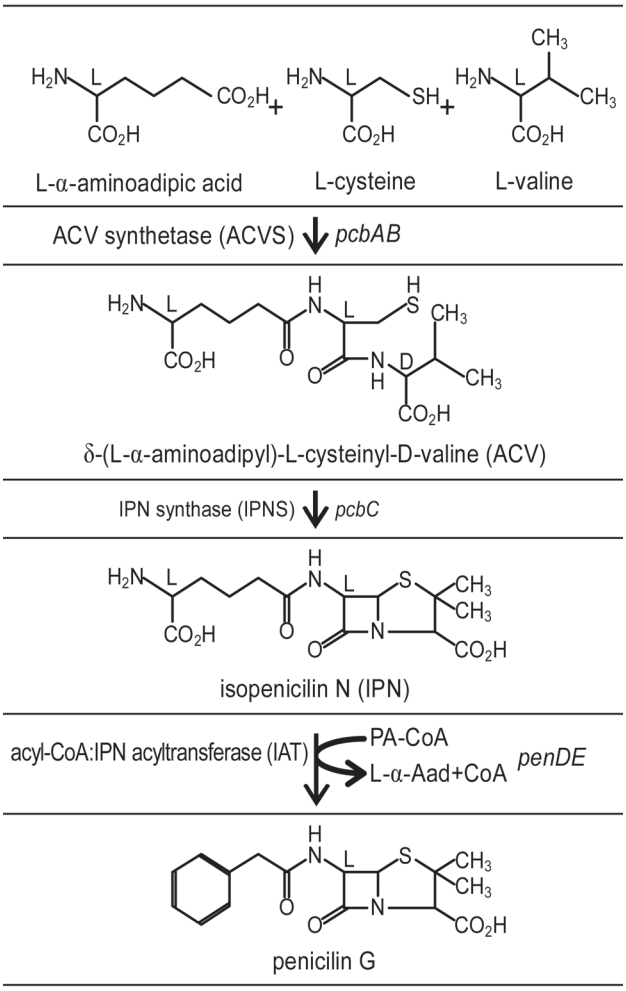

Fig. 1. The pathway of penicillin G biosynthesis, see descriptions in section 2

by the uncommon δ-α binding and the LLD configuration. Other features are the short one-way path of penicillin G biosynthesis and the formation of the bi-cuclic penam nucleus [36, 37]. In primary metabolism the peptides are synthesized in the process of transcription and translation and composed of linearly arranged amino acids combined with peptide bindings formed from the carboxylic- and amino-groups situated in the position α of the amino acids.

4. The role of peroxisomes in penicillin G biosynthesis

Functional peroxisomes (Fig. 2) play a crucial role in the biosynthesis of penicillin G by *P. chrysogenum* [3, 4, 23, 24, 25, 28]. Positive correlations have been reported between the number of large peroxisomes and penicillin G biosunthesis [12, 13, 20, 26, 34, 36]. Two models of peroxisome development have been documented, i.e. *de novo* synthesis from the endoplasmic reticulum and multiplication by fission of pre-existing organelles [3, 4, 9, 10, 20–22, 27, 30, 31]. Mutants blocked in the biosynthesis of peroxisomes have a significant reduced penicillin V production [36]. Overexpression of peroxisome proliferation gene *pex*11 in *P. chrysogenum* results in an increase of both peroxisome numbers and the activity of penicillin V biosynthesis [13]. It was suggested, that the peroxisomal collocation of IAT and PCL provides a clear benefit for the coordination of the last step in penicillin G biosynthesis [19]. When the PSL1 targeting signal is removed from IAT, the enzyme no longer localizes to the peroxisomes, but is diverted to the cytosol and the vacuole. In these cells, penicillin G production by *P. chrysogenum* is completely damaged [23, 36]. Inactivation of autophagy-related degradation of peroxisomes by disruption of *ATG* gene encoding a serine-threonine kinase results in enhanced IAT and IPNS levels and in significantly increased production of penicillin V.

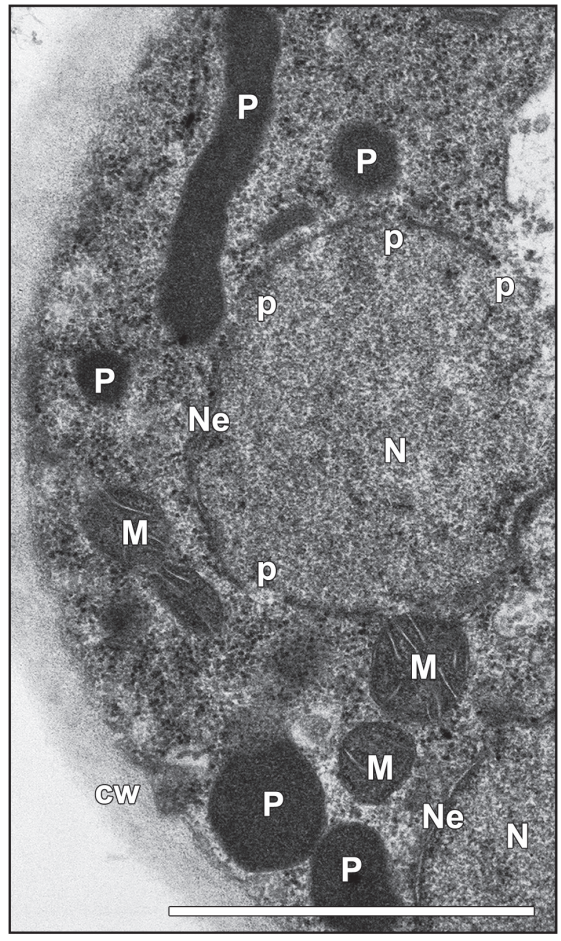

Fig. 2. *P. chrysogenum*, high-penicillin-producing strain, 72 h cultivation, transmission electron microscopy. Note the structural organization of the sub-apical metabolically active non-growing vacuolated cell of the mycelium in penicillin G biosynthesis. Large peroxisomes (P) are characteristically arranged in the peripheral cytoplasm close to the cell wall (cw). The peroxisomes consisting of electron dense protein-reach matrix are associated with numerous mitochondria and spherical interphase nuclei (N). In the nuclear envelope (Ne) pores (p) are visible. Scale bar = 1 μm.

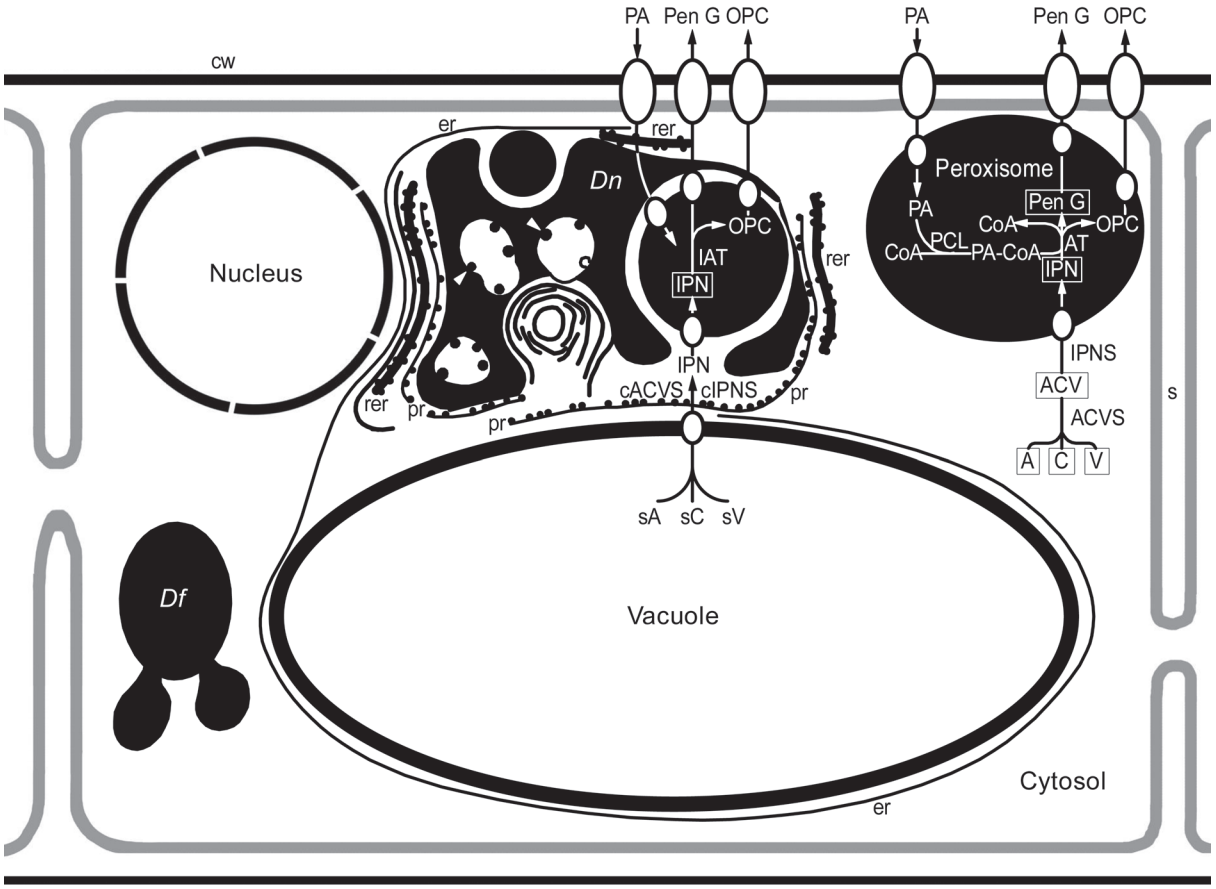

Fig. 3. Schematic arrangement of the pathway of penicillin G biosynthesis in sub-apical productive non-growing vacuolated hyphal cells of *P. chrysogenum* (high-yielding strain at the high activity of penicillin G biosynthesis) is presented. The cytosolic enzymes cACVS and cIPNS are concentrated in the cytoplasm at the polyribosomes surrounding the peroxisome encompassed IAT and PCL. Such a collocation of organelles and enzymes may facilitate the continuous and efficient biosynthetic flux and the immediate conversions in penicillin G biosynthesis. The precursor amino acids can be immediately supplied from the vacuole (sA, sC, sV) and from the cytosol (A, C, V) as well as from the fermentation broth (from the corn steep liquor). Penicillin G (end-product) and 6-oxopiperidine-2-carboxilic acid (OPC) – side-product of antibiotic biosynthesis are transported from the productive cells into the medium. *De novo* (*Dn*) peroxisome formation (P) is presented. Most peroxisomal electron opaque proteins are synthesized on free polyribosomes (pr) and imported directly from the cytosol into the peroxisomes. Note the membrane combined electron opaque proteins concentrated in foci that bud off into the space of the peroxisomal matrix (arrow heads).The peroxisomal volume seems to be an important feature in penicillin G production. The increased membrane surface may promote Penicillin G biosynthesis as it can increase the influx of IPN and PA from the cytosol and improve the transport of penicillin G from the peroxisome into the cytosol. Peroxisome fission (*Df*) of pre-existing organelles is shown.

5. Compartmentalization in penicillin G production

Localization of the pathway of penicillin G biosynthesis in *P.* chrysogenum cells has been studied using transmission electron microscopy [15], immuno-gold electron microscopy [17, 23, 24, 25, 35], cell fractionation [16, 24, 35], biochemical and genetic methods [19]. These experimental programs allowed establish the cellular organization in penicillin G biosynthesis. The compartmentalization of the pathway of penicillin G biosynthesis is depictured in Fig. 3. ACVS and IPNS are localized in the cytosol [35] and PCL and IAT are peroxisomal enzymes [8, 25, 35]. Our ultra-structural analyses suggest that in the cells of the sub-apical productive non-growing vacuolated cells of the mycelium the peroxisomes are frequently arranged in the peripheral cytoplasm and at the vacuoles (data not shown). Using the methods of immuno-gold labeling of IAT such an arrangement of peroxisomes was also visible in the mycelial cells of the industrial strain of *P. chrysogenum* [8, 35]. The observed arrangement of peroxisomes may increase the enzyme supplying efficacy in penicillin G biosynthesia from the medium and from the cytosol as well as from the vacuolar pool. Uptake of amino acids from the medium may contribute to penicillin G biosynthesis. It was reported that the vacuole may play an auxiliary role in supply of precursor amino acids and intermediates in penicillin G biosynthesis [33].

6. Industrial strains

Intense classical strain improvement has yield industrial *P. chrysogenum* strains producing high titers of penicillin G. These strains contain up to eight copies

of penicillin G biosynthetic gene cluster consisting of three genes *pcbAB*, *pcbC* and *penDE*. The *phl* gene is not part of the penicillin G biosynthetic gene cluster. These industrial strains exhibit elevated transcription of genes involved in penicillin G biosynthesis [36, 37]. In *Penicillium chrysogenum*, the biosynthetic pathway of *P. chrysogenum* is compartmentalized [6, 18] and is suggested to take place mainly in sub-apical productive non-growing vacuolated hyphal cells [29]. Metabolic engineering has proven to be a rational alternative to classical strain improvement [32].

7. Detoxification hypothesis of penicillin G biosynthesis

During industrial penicillin G production, PA is fed to the fermentation broth in small amounts to avoid toxic side reactions to the cells of the mycelium [19]. In penicillin G biosynthesis PA is taken up from the fermentation broth and inside of peroxisomes coupled to 6-APA. It suggests that penicillin G biosynthesis by the industrial strains is a cellular detoxification process. In agreement with this suggestion, it was described that the formation of different secondary metabolites has implications for various cellular processes, including cellular defense [11]. In this processes peroxisomes abundantly perform detoxification reactions of toxic to the cytoplasm organic compounds and metabolites leading to the production of secondary metabolites with very interesting biological or pharmaceutical activities [19]. Peroxisomes are organelles that often proliferate in response to compounds that they metabolize [31].

8. Conclusions

The overproduction of penicillin G by industrial strains of *P. chrysogenum* is associated with a strictly adjusted cellular organization. Peroxisomes are involved in penicillin G biosynthesis. The enzymes ACVS and IPNS of the pathway of penicillin G biosynthesis are located in the cytosol. IAT and PCL are peroxisome encompassed enzymes. The peroxisomes are frequently arranged at the periphery of the cytoplasm and around the vacuoles. Such a location enables a sufficient and continuous enzyme supply in penicillin G biosynthesis from the fermentation broth and from the cytosol. The vacuole may play an ancillary role in the supply of precursor amino acids, and in the storage of intermediates of penicillin G biosynthesis. The described results support the phenylacetic acid detoxification hypothesis of penicillin G biosynthesis.

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