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ANTIBIOTIC BIOSYNTHESIS AND SECONDARY METABOLISM IN HIGH-YIELDING STRAINS OF *STREPTOMYCES*, *PENICILLIUM CHRYSOGENUM* AND *ACREMONIUM CHRYSOGENUM*

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Antibiotic biosynthesis and secondary metabolism in high-yielding strains of *Streptomyces*, *Penicillium chrysogenum* and *Acremonium chrysogenum*

Abstract: In this article, the secondary metabolism as a basis for antibiotics production by industrial strains of *Streptomyces*, *Penicillium chrysogenum* and *Acremonium chrysogenum* is discussed. Images from transmission electron microscopy reveal some important features of the mycelial cells which are related to antibiotics biosynthesis. This discovery is important for further industrial strain improvement and has economic significance. Possibilities of new strategies for antimicrobial treatment are discussed.

Biosynteza abtybiotyków i metabolitów wtórnych przez wydajne szczepy *Streptomyces*, *Penicillium chrysogenum* i *Acremonium chrysogenum*

Streszczenie: Rola wtórnego metabolizmu w biosyntezie antybiotyków wytwarzanych przez szczepy przemysłowe z rodzaju *Streptomyces* oraz *Penicillium chrysogenum* i *Acremonium chrysogenum* jest dyskutowana. Obrazy z transmisyjnej mikroskopii elektronowej wykazują ważne cechy komórek grzybni związane z wysokowydajną biosyntezą antybiotyków. Odkrycie to pozwala na dalsze zwiększanie antybiotycznej wydajności szczepów przemysłowych i ma znaczenie ekonomiczne. Przedmiotem niniejszej publikacji jest omówienie możliwości opracowania nowych strategii zwalczania chorób zakaźnych.

Key words: antibiotics, biosynthesis, cellular features, fungi imperfecti, *Streptomyces*
Słowa kluczowe: antybiotyki, biosynteza, cechy komórkowe, fungi imperfecti, *Streptomyces*

1. Introduction

Antibiotics are secondary metabolites produced by different microorganisms, including *Streptomyces* spp. and *Penicillium chrysogenum* as well as *Acremonium chrysogenum* (*Cephalosporium chrysogenum*). High yielding strains of these microorganisms are widely used for antibiotic production on an industrial scale [1, 2, 4, 13]. Large scale of antibiotics production is a result of industrial strain improvement. Antibiotics have saved millions of human beings from the annihilation. Below we discuss different pathways and aspects of antibiotics biosynthesis.

This article is designed to overview details concerned with the basic pathways of antibiotic biosynthesis by *Streptomyces* spp. and fungi imperfect, including industrial strain improvement. Further purpose is to discuss the structural organization of industrial mycelia related to antibiotic biosynthesis. Other goals are to overview efforts to obtain modified antibiotics and to develop new strategies for antimicrobial treatments.

2. Industrial strain improvement

The prokaryotic microorganisms *Streptomyces* and the eukaryotic *P. chrysogenum* as well as *A. chrysogenum* are used for antibiotic biosynthesis on a large scale. These improved and selected high-yielding strains have been used for more than 75 years for antibiotic

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production. Classical strain improvement has yield industrial strains that produce high amounts of antibiotics. Large scale of antibiotics production is a result of industrial strain improvement, including numerous mutations and selections. Metabolic engineering has also proven to be a rational alternative to classical strain improvement [20]. Some *P. chrysogenum* strains contain up to eight copies of the penicillin G biosynthetic gene clusters, i.e.: *pcbAB* gene encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), *pcbC* gene encoding Isopenicillin N synthase (IPNS) and *penDE* gene encoding acyl-Co A:isopenicillin N acyltransferase (IAT). The phenylacetyl-CoA ligase (PCL) encoded by *phl* gene is not a part of the penicillin G biosynthetic gene cluster. A large number of genomic and transcriptional analysis of strain lineages have lead to the identification of numerous alterations in industrial strains, such as the amplification of penicillin G biosynthetic gene cluster, elevated transcription of genes involved in biosynthesis of this antibiotic and its amino acid precursors as well as genes encoding peroxisome proliferation [24, 25]. Biosynthesis and secretion of penicillin G, cephalosporin C are compartmentalized processes located in sub-apical, productive, non-growing cells of the hyphae [3, 6–12, 18, 21]. Cellular organization of industrial *Streptomyces* strain is currently not fully known.

3. Pathways of antibiotic biosynthesis by *Streptomyces* spp.

Antibiotics are secondary metabolites [26] produced by numerous bacteria and fungi. The secondary metabolism is characterized by unusual short (one-way) biosynthetic pathways leading to uncontrolled large scale production of end-products which are stored in cells of the producer or excreted to the cellular environment. Accumulation of secondary metabolites suggests that the productive cells are inefficient in regulation of the biosynthesis of these substances. In view of what has been known about the genetic control of the primary metabolism regulation it would be of interest to find out why the regulation of secondary metabolites biosynthesis is not controlled more strictly. Pathways of secondary metabolism branch out from the main roads of the primary metabolism, such as: fatty acid metabolism, amino acid metabolism, carbohydrate metabolism, purine and pyrimidine metabolism, aromatic biosynthesis (shikimic acid), methyl groups arising from the C1 pool. The branching point of both metabolisms is the uptake of one or few intermediates from one or more pathways of the primary metabolism. Next these intermediates are combined to secondary metabolites. Each of these primary metabolic categories

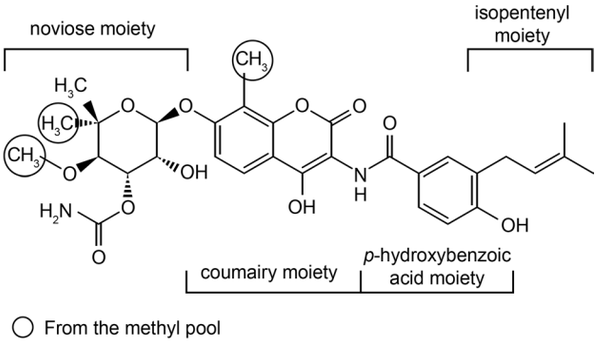

Fig. 1. Primary metabolic sources of novobiocin are pictured

as a source of antibiotic will be discussed in this paper. But first, it should be clear that some antibiotics originate from more than one of these metabolic sources. A good example is novobiocin [26] composed of moieties derived from the following metabolic sources (Fig. 1). The noviose moiety of novobiocin is derived without cleavage from the carbon chain of glucose (carbohydrate metabolism). The methyl groups are mainly derived from the methyl pool, which in turn originates from the methyl group of methionine (C1 pool). The nitrogen of the carbamyl group combined with the noviose ring of novobiocin arises from metabolic nitrogen, probably from glutamine (nitrogen metabolism). The isopentenyl moiety attached to p-hydroxybenzoate is derived from mevalonic acid, which in turn is supplied from either acetate or leucine (isopentyl metabolism). The 3-amino, 4-hydroxycoumarin moiety is derived from tyrosine, which in turn arises from the common aromatic pathway via shikimic acid (aromatic amino acid metabolism). The p-hydroxybenzoate moiety of the antibiotic is derived from the common aromatic pathway via shikimic acid (shikimic acid metabolism).

Short fatty acids biosynthesis in the primary metabolism delivers precursors for antibiotic biosynthesis in the secondary metabolism. Repeated condensation of active acetate and malonate units leads to formation of β-polyketomethylene chains (Fig. 2). In primary metabolism these compounds are reduced to form fatty acids. It was demonstrated that by "head-tail" condensation of acetyl-CoA and malonyl-CaA units numerous secondary metabolites, e.g. mycotoxines, alkaloids, terpenes, steroids, glycosides, including antibiotics can be produced [26]. This last group is represented among others by the following antibiotics (Fig. 3): griseofulvin, erythromycin, tetracyclines, anthracyclines, nystatin and curvularin. The starter molecule of β-polyketomethylene synthesis is not always acetyl-CoA. For example, in tetracycline biosynthesis (Fig. 4) the starter molecules are acetyl-CoA, malonyl-CoA and malonamyl-CoA. The β-polyketomethylenes occur in the cells of the producers always bound to the enzymes. In the secondary metabolism the β-polyketomethylene

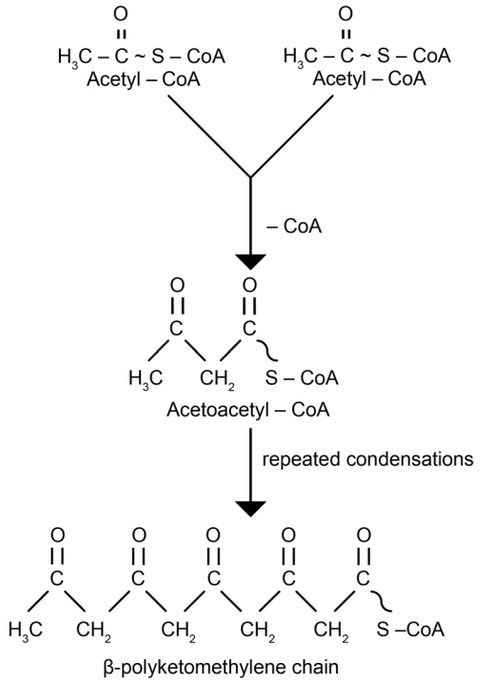

Fig. 2. Biosynthetic pathway of the β-polyketomethylene chain

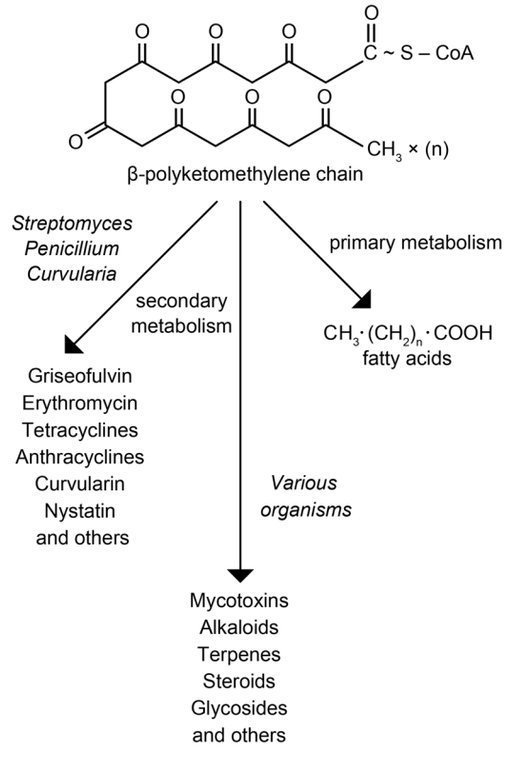

Fig. 3. Metabolic origin of antibiotics and other secondary metabolites

chain undergo in the productive cells of *Streptomyces* spp. numerous further modifications, such as methylation, ring closure, oxidation, chlorination, amination, and others, leading to the biosynthesis of numerous secondary metabolites, such as tetracyclines.

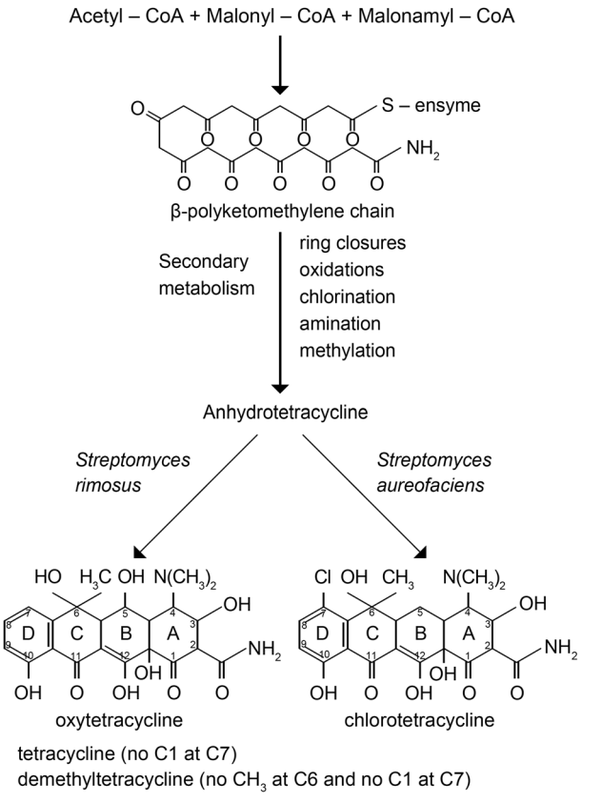

Fig. 4. Scheme of tetracyclines biosynthesis

Some of secondary metabolites possess very strong antibacterial activities. For example, introduction of double and triple bounds results in building of polyene antibiotics (nystatin, amphotericin), which are widely distributed among streptomycetes. Finally, cyclization of the intermediates leads to formation of large lactones (macrolide antibiotics). Through repeated cyclization polyaromatic antibiotics can be produced, e.g. tetracyclines (Fig. 4) and anthracyclines.

Peptide antibiotics are produced by bacteria including *Streptomyces* spp. and fungi. The presence of unusual amino acids of D-configuration and unusual chemical bounds, e.g. δ-α peptide bound in the molecule of peptide antibiotics are characterizing these compounds as secondary metabolites. Other feature of secondary metabolites with strong antimicrobial activity is the nonribosomal biosynthesis and cyclization of the peptide chain. For example, the cyclic peptide of gramicidin S is composed of ten amino acids, including two L-valines, two L-leucines, two L-prolines, two L-ornithines and two D-phenylalanines (the last two amino acid are unusual in the primary metabolism). Bacitracin is a mixture of related cyclic peptides. This antibiotic is composed of twenty aminoacids, including four unusual amino acids: D-glutaminic acid, D-asparagine, D-ornithine, D-phenylalanine and the thiazoline moiety from L-cysteine and L-isoleucine. Polymyxin B_1 contains unusual D-phenylalanine and L-diaminobu-

tyric acid. *Streptomyces* are privileged in the biosynthesis of peptide antibiotics. *Streptomyces antibioticus* is a producer of actinomycin D containing six unusual amino acids, i.e.: two D-valines, two sarcosines and two N-methyl-valines. *S. lavendulae* is a producer of etamycin, a cyclic peptide antibiotic containing D-leucine, D-allo-hydroxyproline, sarcosine, α-phenylsarcosine and N,β-dimethyl-L-leucine. *S. clavuligerus* is a producer of clavulanic acid which is a cyclic peptide. Other cases are the cyclic β-lactam antibiotics structuraly composed of L-cysteine and D-valine.

Sugars as constituents of antibiotics are represented by numerous sugar containing compounds, such as aminoglycoside antibiotics: streptomycin, kanamycin A, neomycin, and others; anthracyclines; purine-antibiotics for example puromycin; polyene antibiotics: nystatin, amphotericin B and others. Antibiotics containing purines or pyrimidines are represented by toyocamycin (from *S. rimosus*), tubercidin (from *S. tubercidicus*), nebularin, cordycepin, psicofuranine and angustmycin A.

4. Compartmentalization in antibiotic biosynthesis by *Streptomyces* spp.

The cellular features of the industrial strains of *Streptomyces* are not yet fully understood. The cellular co-location of enzymes of the pathway of antibiotics biosynthesis should be in the future the subject of intensive investigations. Our studies showed in the cells of *S. vinaceus* L-6 producing viomycin on an industrial scale a large number of organelles filled with electron-dense dark contents (Fig. 5). In the productive mycelial cells of the low-yielding strain *S. vinaceus* 2560 these membranous structures could be observed very rarely. Viomycin is a strongly basic nonribosomal peptide antibiotic with high affinity to heavy metals, visible in electron microscopic images as electron opaque dark material. Therefore, it may be suggested, that in the high-yielding strain the electron-dense content of these organelles is related to the places of viomycin accumulation. It is also supposed that these structures plat a role in the processes of viomycin biosynthesis. We have also observed similar organelles in the cells of other high-yielding *Streptomyces* strains, e.g.: *S. melanochromogenes*, *S. erythreus*, *S. aureofaciens*. Better understanding of the cellular features associated with antibiotic biosynthesis on an industrial scale will deliver further opportunities to increase the production efficiency of these compounds, as was the case with penicillin G biosynthesis by *P. chrysogenum.*

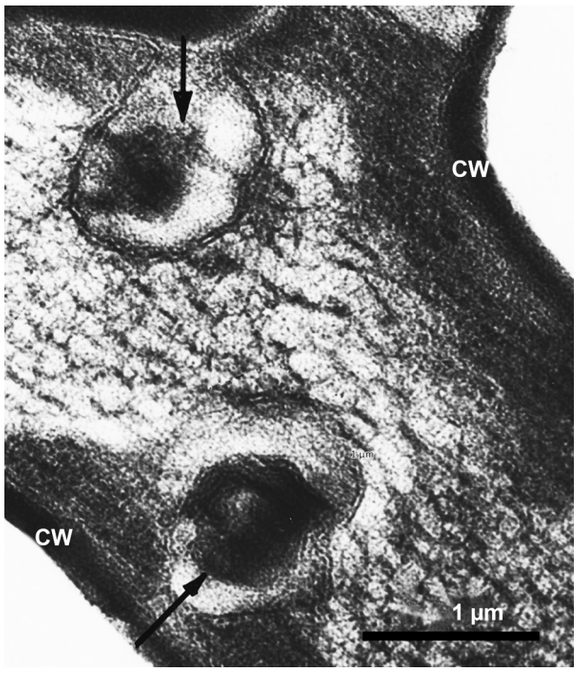

Fig. 5. *Streptomyces vinaceus* L-6
Ultrastructural features of a high-viomycin-yielding cell of the mycelium. Abbreviations: cw– cell wall; Arrows showing organelles filled with electron-dense dark contents.

5. The pathway of penicillin G biosynthesis by *P. chrysogenum*

The biosynthesis of penicillin G consists of at least three enzymatic steps. The enzyme ACVS is involved in condensation of activated amino acids L-α-aminoadipic acid, L-cysteine and L-valine to form the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) [24]. IPNS converts the ACV to isopenicillin N (IPN). The enzymes PCL and IAT are involved in the last step of penicillin G biosynthesis, in which the α-aminoadipyl group of IPN is substituted by a phenylacetyl side chain [24].

6. Compartmentalization in penicillin G biosynthesis by *P. chrysogenum*

Nowadays the compartmentalization in penicillin G biosynthesis by *P. chrysogenum* is better understood [3, 12, 16, 17]. It was suggested that the sub-apical productive non-growing vacuolated hyphal cells are privileged in antibiotic biosynthesis [18]. Based on biochemical features and immune-gold electron microscopy it was shown that ACVS is a cytosolic enzyme [21, 23]. The IPNS was previously found to be associated with membranous organelles [5, 11]. Based on sub-cellular fragmentation, immune-gold electron microscopy and biochemical features of the enzyme van der Lende *et al.* [23] suggested that IPNS is a soluble cytosolic enzyme, although its activity in cell free extracts seems to be stimulated by sonification [21]. It suggests a partial

cellular compartmentalization and concentration of IPNS, e.g. as cytosoloc enzymes around the peroxisomes. Our findings show that at the industrial activity of penicillin G production the enzyme IPNS is frequently concentrated in the cytoplasm between the membranes of polyribosomes surrounding the peroxisomes [6, 7]. The enzymes PCL and IAT are located in peroxisomes [15, 23]. Such a collocation of the cytosolic ACVS and IPNS with the peroxisomal PCL and IAT may increase the IAT supplying efficacy. Moreover such a collocation may also facilitate the continuous and efficient biosynthetic flow of molecules and the immediate conversion in penicillin G biosynthesis. Formation of the IPN (the β-lactam nucleus) takes place in the cytosol. IPN is next transported into the peroxisomes where the L-α-aminoadipyl side chain of IPN is exchanged for a phenylacetyl group by IAT. In this reaction the side chain precursor has to be activated by PCL before the translocation occurs [14]. These results indicate that the last two steps in penicillin G biosynthesis are located in peroxisomes, where this antibiotic is synthesized, accumulated and entrapped. Functional peroxisomes play a crucial role for the efficiency of penicillins production by industrial strains [14, 15, 22]. High penicillin G producing strains show increasing numbers of peroxisomes [14, 22, 24]. Overexpression of the peroxisome proliferation gene *pex*11 in *P. chrysogenum* results in an increase of both peroxisome numbers and in the activity of penicillin biosynthesis. The productive mycelia cells of high penicillin G producing strains show increasing numbers of large peroxisomes mainly at the period of the intensive antibiotic biosynthesis [1, 6, 7, 22, 24, 25]. *De novo* synthesis of peroxisomes and collocation of large peroxisomes with vacuole as well as peroxisomes multiplication by fission are significant features of the high productive non-growing mycelial cells. The productive hyphal cells of the high-yielding strain of *Penicillium chrysogenum* PQ-96 exhibited numerous peroxisomes (Fig. 6) frequently arranged at the periphery of the cytoplasm and around the large vacuoles. The peroxisomes are abundantly accompanied by ACVS and IPNS which are entrapped in the cytoplasmic areas located between the surface of peroxisomes and polyribosomes surrounding the peroxisomes. Such a collocation of the cytosolic ACVS and IPNS with the peroxisomal PCL and IAT may increase the intermediate supplying efficacy in penicillin G biosynthesis from the fermentation medium and from the cytosol as well as from the vacuolar pool. Our findings show that in the process of penicillin G production on a lagge scale the structurally grouped organelles build a well organized "assembly line" consisting of cytosolic and membrane encompassed enzymes, substrates, intermediates, precursors, side- and end-products [6, 7].

Secretion of penicillin G from the interior of peroxisomes first across the plasma membrane and then through the cellular membrane of the industrial mycelia of *P. chrysogenum* is at present poorly understood [24]. It is unknown whether IPN and penicillin G transport over the membrane of peroxisomes requires active transport or utilizes peroxisomal pore proteins. It was suggested that the abundant pexophagy and exocytosis should be currently considered as putative alternative for active secretion by the ABC transporters. The results of our previous experiments show that the abundant vacuolar pexophagy, i.e. the autophagy-related degrada-

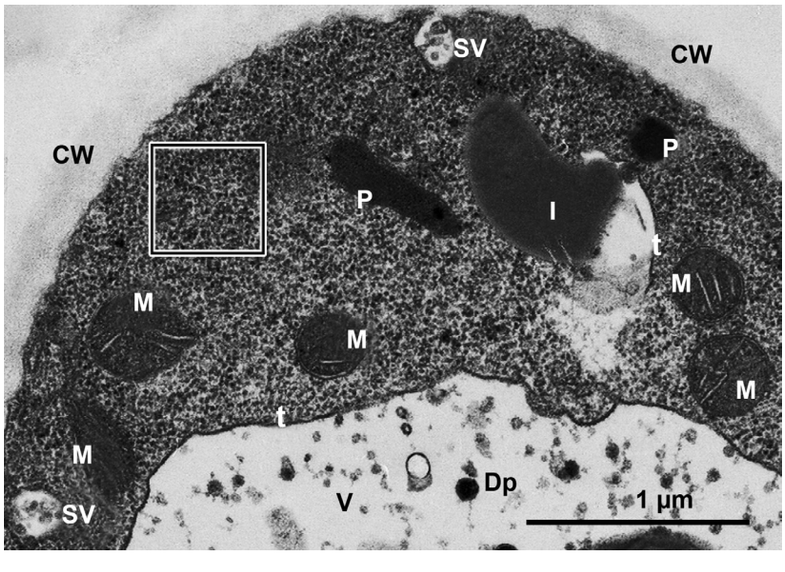

Fig. 6. *Penicillium chrysogenum* PQ-96, high-penicillin-yielding strain
Ultrastructural features of a productive cell of the mycelium. Abbreviations: cw – cell wall, sv – secretion vesicles, M – mitochondria, P – peroxisomes, l – lipid body, t – tonoplast, V – vacuole, Dp – degradation products of peroxisomal matrix, square – ribosomes and a polyribosome is visible.

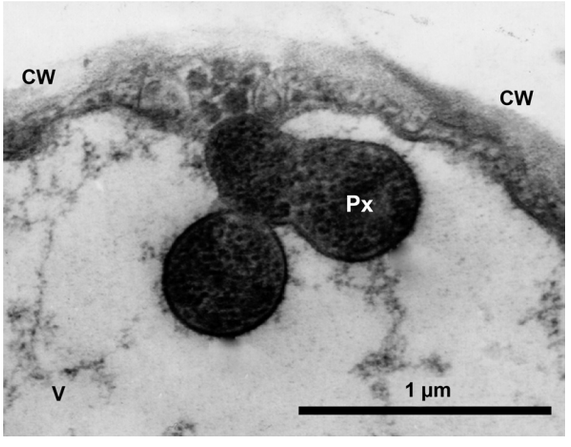

Fig. 7. Penicillium chrysogenum PQ-96, high-penicillin-yielding strain
Abbreviations: cw – cell wall, V – vacuole, Px – vacuolar pexophagy.

tion of peroxisomes takes place mainly in mature and late sub-apical cells of *P. chrysogenum* during the intensive increase of antibiotic performance in the supernatant of the fermentation broth. Secretion of penicillin G from the peroxisomes across the plasma membrane of the mycelia cells of *P. chrysogenum* is poorly understood [24]. The results of our previous experiments show that the abundant vacuolar pexophagy of large peroxisomes (Fig. 7) combined with vacuolar budding and the presence of a large number of vacuolar vesicles which fuse with the plasma membrane are the most important structural features characterizing the productive cells of the industrial strains of *P. chrysogenum* [9, 10]. Therefore, we suggest that in industrial-scale secretion of penicillin G the pexophagy and exocytosis should be currently considered as a putative alternative for active secretion by the ABC transporters. Despite of numerous efforts, at present, the lack of clear involvement of any of these ABC transporters (van den Berg 2001, Patent description WO 2001/32904) in secretion of penicillin G is intriguing and may suggest that the secretion of penicillin G by industrial mycelia does not proceed through the classical ABC pumps. In the industrial cultures the overproducing strains secrete 40–55 g of penicillin G per liter of the fermentation medium and in this case the ABC transporters seem to be inefficient. The extracellular level of penicillin G is tenfold higher than the intracellular concentration of this antibiotic [22]. It suggests an active secretion mechanism [24].

7. The pathway of cephalosporin C biosynthesis by *A. chrysogenum*

The pathways of penicillin and cephalosporin biosynthesis are related. In *Penicillium* IPN is converted to penicillin G [24]. The formation of IPN is a branch point of the pathways of penicillin and cephalosporin biosynthesis. In *Acremonium* and other cephalosporin producers IPN is converted to penicillin N (PEN) by an epimerization CefD1-CefD2 system [19]. The next step in cephalosporin 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 PEN expandase (deacetoxycephalosporin C synthase – DAOCS) – encoded by the *cefEF* gene. The following step is conversion of deacetoxycephalosporin C (DAOC) to deacetylcephalosporin C (DAC) by DAC synthase. The last step of cephalosporin C biosynthesis is the transfer of an acetyl-moiety from the acyl-CoA to hydroxyl group on the sulfur containing ring of DAC, this reaction is catalyzed by the acyl-CoA:DAC acyltransferase – encoded by *cefG*. Cephalosporin C is a peptide secondary metabolite with high antibacterial activity.

8. Compartmentalization in cephalosporin biosynthesis by *A. chrysogenum*

In *Acremonium* the CefD1-CefD2 epimerization system converting isopenicillin N to penicillin N (D-isomer of IPN) is located (entrapped) in peroxisomes [12, 13]. All other enzymes of the pathway of cephalosporin C biosynthesis, i.e. DCVS, IPNS, DAOCS, DACS, DAC-acetyltransferase are suggested to be cytosolic enzymes [21]. The results of our previous experiments exhibit in the productive non-growing cells of the industrial mycelium of *A. chrysogenum* a large number of peroxisomes which are accompanied by mitochondria and polyribosomes. Small peroxisomes were also rarely visible in the cells of the low cephalosporin producing strain of *A. chrysogenum*. This results support strongly the participation of peroxisomes in cephalosporin biosynthesis [8].

9. The future of antibiotic therapy

Over many decades, the always increasing resistance of different pathogens to antibiotics was forcing as to modify their chemical structure to obtain new compounds with improved antimicrobial activity. So far, we always modify the antibiotics to get more efficient antimicrobial compounds but as response to our modifications the pathogens are doing exactly the same modifying their mechanism of resistance to not accept our cure. Nevertheless, the search for new antibiotics that could be medically used is at present the order of the day. The future of antibiotics must be protected by the correct and rational medical application. Moreover, new strategies of medical treatment should

be developed, e.g. the antibiotics used on a large scale for a long time with decreased antimicrobial activity should be withdrawn from treatment for few years and then tested for repeated use. Scientific reports and discussions between experts point to the suitability of such procedures [24, 25]. New strategies of medical treatment mentioned above should be developed.

10. Conclusions

Further experimental programs should be conducted to deliver more details related to biosynthesis and secretion of antibiotic from the industrial mycelia of *Streptomyces* spp., *P. chrysogenum* and *A. chrysogenum*. Better understanding of the cellular compartmentalization leads to modern technologies of biosynthesis with increased antibiotic efficiency which has economical importance. Further efforts should be taken to obtain modified natural, semisynthetic and synthetic antibiotics. Cell-free technologies using immobilized enzymes of the pathways of antibiotics biosynthesis and the molecular docking seems to be the future in this field. Classical strain improvement and genetic engineering provides further possibilities to elaborate new strategies of antimicrobial treatments.

Acknowledgements

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