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SHORT COMMUNICATION

Pexophagy in Penicillin G Secretion by *Penicillium chrysogenum* PQ-96

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Abstract

Penicillin G oversecretion by *Penicillium chrysogenum* PQ-96 is associated with a strictly adjusted cellular organization of the mature and senescent mycelial cells. Abundant vacuolar phagy and extended cellular vacuolization combined with vacuolar budding resulting in the formation of vacuolar vesicles that fuse with the cell membrane are the most important characteristic features of those cells. We suggest as follows: if the peroxisomes are integrated into vacuoles, the penicillin G formed in peroxisomes might be transferred to vacuoles and later secreted out of the cells by an exocytosis process. The peroxisomal cells of the mycelium are privileged in penicillin G secretion.

Key words: *Penicillium chrysogenum*, penicillin G, secretion

The last two steps in penicillin G biosynthesis are located in the peroxisomes. Secretion of this antibiotic from the peroxisomes across the plasma membrane of *Penicillium chrysogenum* is poorly understood (Weber *et al*., 2012). This experimental study was designed to provide details supporting the hypothesis that pexophagy (autophagy of peroxisomes) is involved in the large-scale secretion of penicillin G from the mycelial cell of *P. chrysogenum* PQ-96.

In this experimental program the high penicillin-producing strain *P. chrysogenum* PQ-96 was examined. Activity of penicillin G produced by this strain is described in Fig. 1. For comparative ultra-structural analyses the low-penicillin-producing strain *P. chrysogenum* Q-176 was investigated (Fig. 2). The examined strains were grown in complex media (Kurzątkowski *et al*., 2014a) and the antibiotic assay was carried out as described previously (Garcia-Estrada *et al*., 2007). The preparation for transmission electron microscopy and immunoelectron microscopy was performed as described previously (Kurzątkowski *et al*., 2014a). The ultrathin sections were examined under a transmission electron microscope JEOL, JEM 1220 (Tokyo, Japan).

The lack of clear involvement of any of these ABC transporters (van den Berg, 2001; Patent description number WO 2001/32904) in secretion of penicillin G is intriguing and may indicate that the secretion of this antibiotic in the overproducing strains does not proceed through the classical ABC pumps. At present, new secretion pathways, *e.g.* the secretion by exocytosis may have been implemented in the high-penicillin producing strains (Martín *et al*., 2010). Although, fusion of the vacuoles to the plasma membrane by an exocytosis process is possible, there is currently no evidence in the literature to support that this might be a major mechanism of penicillin G secretion (Martin *et al*., 2010).

In fed-bath cultures the industrial strains secrete 40–55 g of penicillin G per liter of the liquid fermentation medium. The knowledge concerned with the cellular arrangements in penicillin G overproduction is important for further strain improvement, which is of great economical importance. Compartmentalization in penicillin G biosynthesis by *P. chrysogenum* PQ-96 was described previously (Kurzątkowski *et al*., 2014a; 2014b). Our studies have proven that overproduction of penicillin G is associated with strongly adjusted mycelial and cellular organization (Fig. 1a-d, Fig. 2i-h, Fig. 3e-h). The productive mycelial cells of the high-yielding strain exhibited numerous large peroxisomes frequently arranged at the periphery of the cytoplasm and around the vacuoles including vacuolar invaginations. The surveys of a large number of hyphal sections

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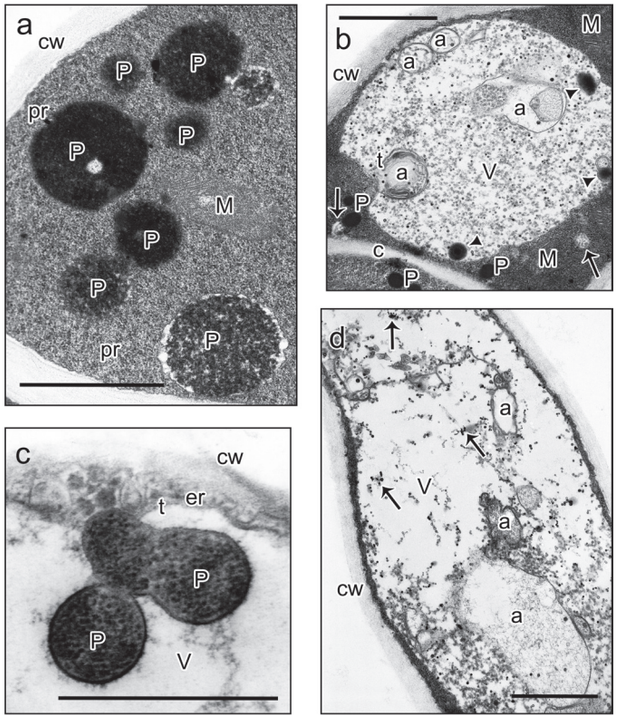

Fig. 1. *P. chrysogenum* PQ-96, 72 h culture, high-penicillin-producing strain, activity of penicillin G biosynthesis (U/ml): total yield at 72 h of cultivation – 8300 (4.98 g/1000 ml), increase of yield between 48 h and 72 h of fermentation – 4850.

(a-d) Transmission electron microscopy. **(a)** In the sub-apical non-growing productive cell numerous electron opaque peroxisomes (P) up to 1.0 µm in diameter can be observed. **(b)** Sub-apical productive cell of the mycelium is visible. Note the degrading organelle located in invaginations of the tonoplast (t) into a vacuole (V). Vacuolar engulfed pexophagy (arrow heads) is seen. It is a characteristic feature of the hyphal cell at the highest activity of penicillin G secretion. At the cell wall (cw) and the cross wall (c) vacuolar vesicles packed with organelle debris are visible (arrows). Some peroxisomes (P) and mitochondria (M) are located at the vacuole beginning the process of autophagy (a). **(c)** Late sub-apical degrading highly vacuolated hyphal cell. In invaginations of the tonoplast (t) into the vacuole (V) the pexophagy (P) is seen. **(d)** Late-apical cell of the mycelium is seen. In the interior of an extended vacuole (V) the products of organelle-autophagy (a) can be seen (arrows). Scale bar = 1 µm.

led us to the conclusion that the immuno-gold marker of isopenicillin N synthase is abundantly arranged at polyribosomes surrounding the peroxisomes. Such a cellular accumulation of isopenicillin N synthase may enhance the selective, continuous and sufficient substrate supply in penicillin G biosynthesis. It was recently found that δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine tri-peptide is present in the cytoplasm and accumulates in the fermentation medium to concentrations of up to 2 mM (for review see Kurzątkowski *et al.*, 2014a). The affinity of isopenicillin N synthase for this tri-peptide is in the sub-mM range. The high concentration of the tri-peptide in the fermentation broth might explain our unexpected results concerning the localization of isopenicillin N synthase at the periphery of the cytoplasm and in channel-like structures of the cell wall. This location might be a precisely adopted structural arrangement enabling the withdrawal of the tri-peptide from the fermentation broth and from the cytoplasm for the peripherally located isopenicillin N synthase to increase the efficacy and yield in penicillin G biosynthesis. On the contrary, in the mature non-growing hyphal cells of the low penicillin-producing strain *P. chrysogenum* Q-176 the total lack of peroxisomes about 0.1 µm in diameter were visible.

Peroxisomes play a crucial role in the production of penicillin G and cephalosporin C by industrial strains (Kurzątkowski and Gębska-Kuczerowska, 2015). High penicillin G producing strains show increasing numbers of large peroxisomes mainly at the period of the intensive antibiotic biosynthesis (van den Berg *et al.*, 2008; Meijer *et al.*, 2010; Weber *et al.*, 2012). The presence of functional peroxisomes remarkably affects the efficiency of penicillin G biosynthesis (van den Berg *et al.*, 2008; Meijer *et al.*, 2010; Bartoszewska *et al.*, 2011). Mutants blocked in the biosynthesis of peroxisomes exhibit a significantly reduced activity of penicillin biosynthesis (Weber *et al.*, 2012).

The results of our experiments exhibit that the abundant vacuolar pexophagy of large peroxisomes combined with vacuolar budding and the presence of numerous vacuolar vesicles which fuse with the plasma membrane are the most important structural features characterizing the non-growing productive cells as well as the late-apical degenerating highly vacuolated cells of the tested industrial strain. This structural arrangement is closely combined with the period of large-scale secretion of penicillin G. Such a cellular organization was not visible in the mature cells of the low-penicillin-producing strain Q-176.

We suggest that the abundant pexophagy of large peroxisomes as well as the vacuolar budding observed in the productive and senescent cells of *P. chrysogenum* PQ-96 might be directly involved in large-scale secretion of penicillin G. In these cellular arrangements the penicillin G formed in peroxisomes might be transferred to vacuoles and late secreted out of the cells by an exocytosis process. The vacuolar pH of about 5 is suitable for the stability of penicillin G. Our discoveries are consistent with the reported positive cor-

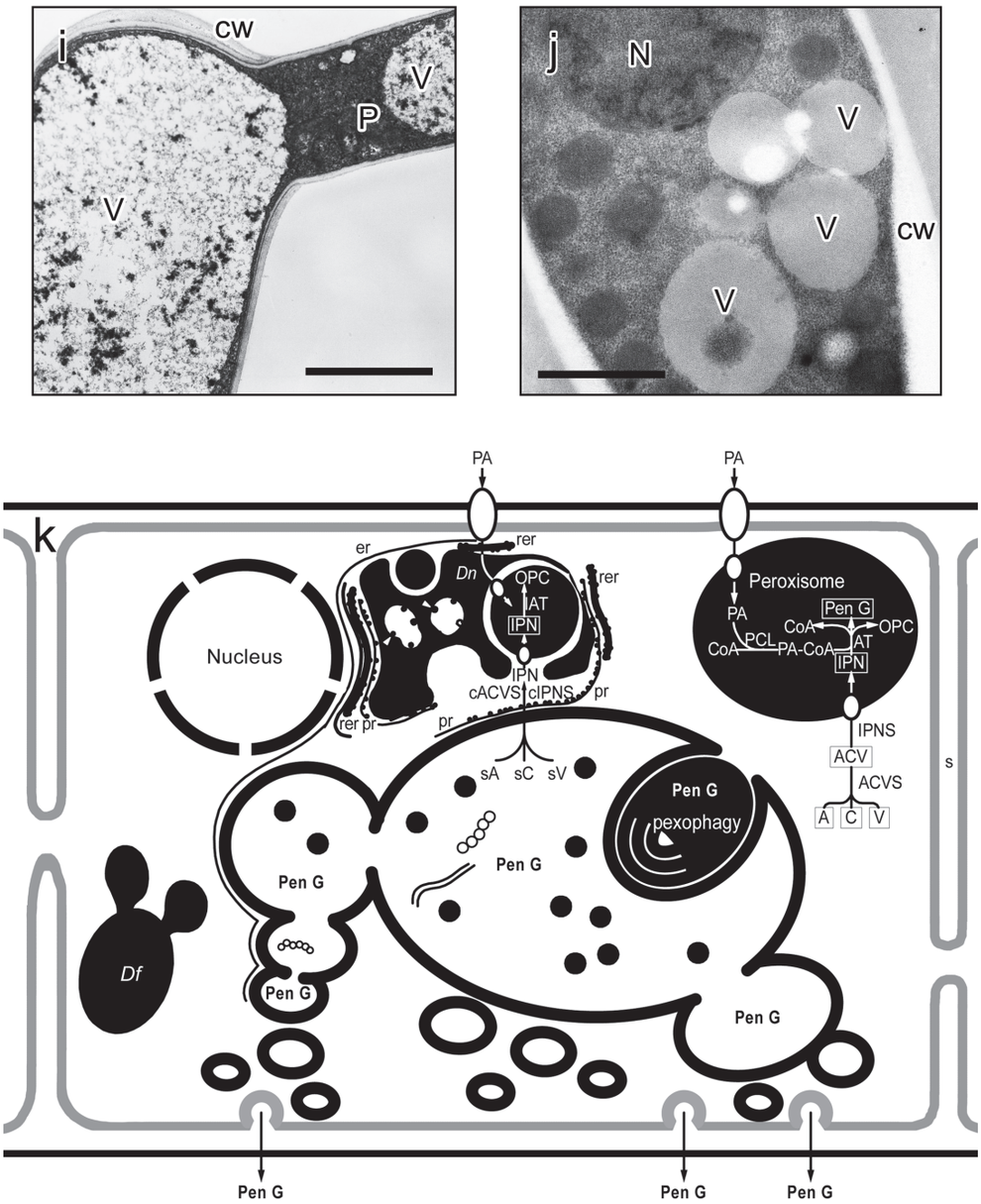

Fig. 2. *P. chrysogenum* Q-176 (low-yielding strain), 72 h culture, activity of penicillin G secretion 70 U/ml.
(i) Transmission electron microscopy. In the sub-apical non-growing branching cell of the hyphae only one small peroxisome is visible. **(j)** Ultrathin sections treated with rabbit immunoserum to isopenicillin N synthase followed by goat anti-rabbit IgG-15 nm gold conjugate showed exceptionally few gold grains. Scale bar = 1 μm. **(k)** A hypothetical overview of penicillin G secretion from sub-apical non-growing vacuolated mycelial cells of *P. chrysogenum* PQ-96 is depictured. The final steps of penicillin G biosynthesis are located in peroxisomes where isopenicillin N is converted to penicillin G. In the process of vacuolar pexophagy penicillin G is transported to the interior of vacuoles. Finally penicillin G is secreted from the vacuoles to the fermentation medium in the process of vacuolar budding followed by fusion of vacuolar vesicles with the cell membrane (exocytosis). *Abbreviations*: A – α-aminoadipic acid; C – L-cysteine; V – L-valine; sA, sC, sV – vacuole sequestered pool of the precursor amino acids; ACV – δ--(L-α-aminoadipyl)-L-cysteinyl-D-valine tri-peptide; ACVS – ACV synthetase; IPN – Isopenicillin N; IPNS – Isopenicillin N synthase; PA – phenylacetic acid; CoA – coenzyme A; PA-CoA – phenylacetyl-coenzyme A; PCL – PA-CoA ligase; AT – acyl-CoA:isopenicillin N acyltransferase; OPC – 6-oxopiperidine-2-carboxilic acid; Pen G – penicillin G; er – endoplasmic reticulum; rer – rough endoplasmic reticulum; pr – polyribosomal membranes; *Dn* – *de novo* synthesis of peroxisomes; *Df* – fission of pre-existing peroxisomes.

relation between the number of large peroxisomes and penicillin G secretion (Meijer *et al.*, 2010), as well as between the extended vacuolization and antibiotic secretion (Sakai *et al.*, 2006).

The novelty of this experimental program is the discovery of essential cellular features associated with the large scale secretion of penicillin G from the mycelium of *P. chrysogenum* PQ-96 to the fermentation

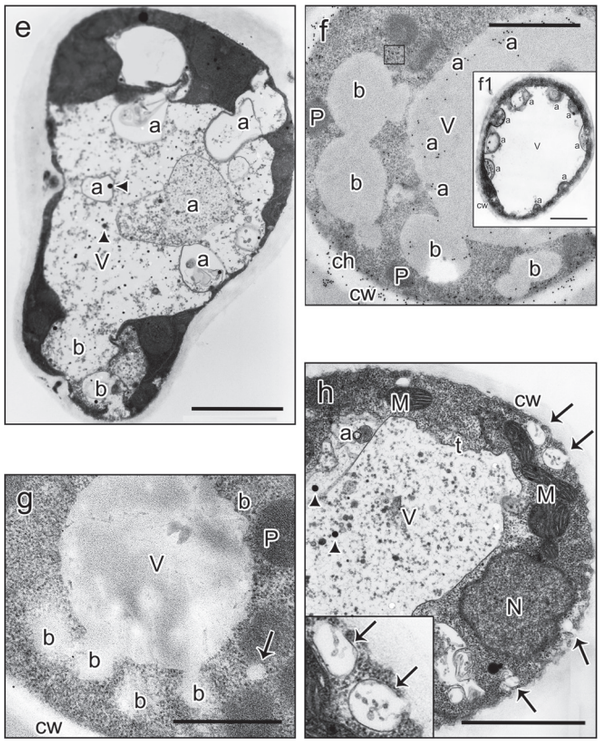

Fig. 3. *P. chrysogenum* PQ-96, 72 h culture. **(e, f1, h)** Transmission electron microscopy.

(e) A sub-apical non-growing productive vacuolated cell of the mycelium. The autophagy (a) of organelles (arrow heads) and budding (b) of a large vacuole (V) is correlated with the highest activity of penicillin G secretion. **(f, g)** Immuno-gold electron microscopy of cytosolic isopenicillin N synthase. **(f)** The marker of the enzyme is associated with peroxisomes (P) including the membranes involved in vacuolar (V) budding (square) and is located in numerous vacuolar invaginations exhibiting places of pexophagy (a) as well as at the periphery of the cytoplasm where the vacuolar vesicles fuse with the plasma membrane and in channels (ch) of the cell wall (cw). Abundant vacuolar budding (b) is seen. **(f1)** At the periphery of a large vacuole (V) numerous structures are visible (a) corresponding to the places of pexophagy shown in position **(f). (g)**. Control sample – ultrathin sections treated with rabbit pre-immunoserum, followed by goat anti-rabbit IgG-15 nm gold conjugate. Immuno-gold localization of not specifically bound antibody showed exceptionally few gold grains. Abundant budding (b) of a vacuole (V) is characteristic for intensive penicillin G secretion. At the vacuolar periphery a peroxisome (P) is visible. A vacuolar vesicle (arrow) of about 100 nm in diameter is seen. **(h)** Section through the sub-apical non-growing vacuolated cell is visible. In the cell at intensive penicillin G secretion, fusion of numerous vacuolar vesicles with the cytoplasm membrane can be observed (arrows). In the interior of these vesicles organelle debris similar to that located in the vacuole (V) are visible. It suggests that after vacuolar pexophagy the vacuolar vesicles are involved in penicillin G secretion. Note the organelle debris (arrow heads) located in the vacuole. Autophagosomes (a) in the vacuole area are visible. Note the mitochondria (M) and the nucleus (N) closely arranged at the massive cell wall (cw). Scale bar = 1 μm.

medium, *i.e.*: abundant vacuolar pexophagy of large peroxisomes, intensive cellular vacuolization, budding of vacuoles, fusion of vacuolar vesicles with the plasma membrane. We have come to the conclusion that in large-scale secretion of penicillin G the pexophagy phenomenon and exocytosis should be currently considered as a putative alternative for active secretion by the ABC transporters.

Acknowledgements

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Literature

Bartoszewska M., Ł. Opaliński, M. Veenhuis and I.J. van der Klei. 2011. The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. *Biotechnol. Lett.* 33: 1921–1931.

García-Estrada C., I. Vaca, M. Lamas-Maceiras and J.F. Martín. 2007. *In vivo* transport of the intermediates of the penicillin biosynthetic pathway in tailored strains of *Penicillium chrysogenum*. *Appl. Microbiol. Biotechnol.* 76: 169–183.

Kurzątkowski W., M. Staniszewska, M. Bondaryk and A. Gębska-Kuczerowska. 2014a. Compartmentalization in penicillin G biosynthesis by *Penicillium chrysogenum* PQ-96. *Polish J. Microbiol.* 63: 399–408.

Kurzątkowski W., M. Staniszewska, M. Bondaryk and A. Gębska-Kuczerowska. 2014b. Penicillin G production by industrial strains of *Penicillium chrysogenum*. *Post. Microbiol.* 53: 366–370.

Kurzątkowski W. and A. Gębska-Kuczerowska. 2015. Compartmentalization in cephalosporin biosynthesis by industrial strains of *Acremonium chrysogenum*. *Post. Microbiol.* 54: 374–379.

Martín J-F., R.V. Ullán and C. Garcia-Estrada. 2010. Regulation and compartmentalization of β-lactam biosynthesis. *Microbial. Biotechnol.* 3: 285–299.

Meijer W.H., L. Gidijala, S. Fekken, J.A Kiel, M.A. van den Berg, R. Lascaris, R.A. Bovenberg and I.J. van der Klei. 2010. Peroxisomes are required for efficient penicillin biosynthesis in *Penicillium chrysogenum*. *Appl. Environ. Microbiol.* 76: 5702–5709.

Sakai Y., M. Oku, I.J. van der Klei and J.A Kiel. 2006. Pexophagy: autophagic degradation of peroxisomes. *Biochim. Biophys. Acta* 1763: 1767–1775.

Weber S.S., R.A. Bovenberg and A.J. Driessen. 2012. Biosynthetic concepts for the production of β-lactam antibiotics in *Penicillium chrysogenum*. *Biotechnol J.* 7: 225–236.