Peroxisomes Are Required for Efficient Penicillin Biosynthesis in *Penicillium chrysogenum* \*†

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In the fungus *Penicillium chrysogenum*, penicillin (PEN) production is compartmentalized in the cytosol and in peroxisomes. Here we show that intact peroxisomes that contain the two final enzymes of PEN biosynthesis, acyl coenzyme A (CoA):6-aminopenicillanic acid acyltransferase (AT) as well as the side-chain precursor activation enzyme phenylacetyl CoA ligase (PCL), are crucial for efficient PEN synthesis. Moreover, increasing PEN titers are associated with increasing peroxisome numbers. However, not all conditions that result in enhanced peroxisome numbers simultaneously stimulate PEN production. We find that conditions that lead to peroxisome proliferation but simultaneously interfere with the normal physiology of the cell may be detrimental to antibiotic production. We furthermore show that peroxisomes develop in germinating conidiospores from reticulate-like structures. During subsequent hyphal growth, peroxisome proliferation occurs at the tip of the growing hyphae, after which the organelles are distributed over newly formed subapical cells. We observed that the organelle proliferation machinery requires the dynamin-like protein Dnm1.

Penicillins (PENs) belong to the group of β-lactam antibiotics that are produced as secondary metabolites by specific actinomycetous bacteria and fungal species (26). For the industrial production of PEN, the filamentous fungus *Penicillium chrysogenum* is used. The biosynthesis of penicillin G (PenG) has been characterized in detail at the genetic and biochemical levels using *P. chrysogenum* and a related fungus, *Aspergillus nidulans*, as model organisms (7, 28). Starting from three amino acids, α-amino adipic acid, cysteine, and valine, PenG is formed in three unique enzymatic conversions (Fig. 1). These amino acids are first condensed to a tripeptide mediated by the function of a nonribosomal peptide synthetase, δ-(1-α-amino-adipyl)-l-cysteinyl-l-valine (ACV) synthetase (ACVS). The resulting tripeptide, ACV, is cyclized by isopenicillin N synthase (IPNS) to form a β-lactam, isopenicillin N (IPN). As a final step, the enzyme acyl coenzyme A (CoA):6-aminopenicillanic acid acyltransferase (AT) replaces the α-aminoadipyl side chain of IPN with a more hydrophobic one. In industrial fermentations, phenylacetic acid (PAA) or phenoxyacetic acid (POA) is applied to produce PenG or penicillin V (PenV), respectively.

In filamentous fungi, the PEN biosynthetic machinery is compartmentalized (Fig. 1). The first two enzymes, ACVS and IPNS, are located in the cytosol (19, 32). As the pH of the cytosol in filamentous fungi is between 6.5 and 7.0 (9, 31), these enzymes are in their optimal physiological surroundings. The AT and phenylacetyl CoA ligase (PCL) enzymes have specific targeting sequences that sort these enzymes to the lumen of their target compartment, the peroxisome (18, 19). The pH of this organelle was shown to be 7.5, which is close to the pH optima of both AT and PCL (31). Apparently, the compartmentalization of these enzymes creates defined microenvironments and enables the generation of favorable substrate and cofactor concentrations for enzyme function.

Peroxisomes (belonging to the family of microbodies) are ubiquitously present in eukaryotic cells. They typically consist of a protein-rich matrix surrounded by a single membrane and are 0.1 to 1 μm in size. Although their function is often species and cell type specific, two widely distributed functions can be distinguished, namely, \( \text{H}_2\text{O}_2 \) metabolism and β-oxidation of fatty acids (for reviews, see references 25, 29, and 30). Muller et al. (18, 19) demonstrated the role of peroxisomes in PEN biosynthesis for the first time. Subsequently, it was speculated that a correlation may exist between the volume fraction of these organelles and PEN production rates (18, 27). This speculation was reinforced by Kiel and colleagues (13), who showed that the artificial proliferation of peroxisomes via the overexpression of the *pex11* gene was associated with a 2- to 3-fold increase in PEN production rates. Here we further elaborate on these studies and show that peroxisomes de facto are required for efficient PEN biosynthesis in *P. chrysogenum*. In addition, we present details on the origin and subsequent partitioning of the organelles over newly formed subapical cells during hyphal development.

MATERIALS AND METHODS

Strains and growth conditions. The *P. chrysogenum* strains used in this study are listed in Table 1. NRRL1951, Wis54-1255, and DS17690 derivatives were
grown, as described previously (15), on PEN production (PP) medium supplemented with 0.25% (wt/vol) PAA or POA. In specific experiments, 0.1% oleic acid and 0.05% Tween 80 were also added to enhance peroxisome proliferation. Growth was done at 25°C at 200 rpm for a maximum of 7 days. Regeneration agar [6.0 ml 87% glycerol, 7.5 ml beet molasses, 5.0 g yeast extract, 18 g NaCl, 0.05 g MgSO$_4$·7H$_2$O, 0.06 g KH$_2$PO$_4$, 0.1 mg CuSO$_4$·5H$_2$O, 0.25 g CaSO$_4$·2H$_2$O, and 20.0 g Oxoid agar per liter] was used to generate conidiospores from each strain. To identify and characterize non-oleate-utilizing mutants, minimal medium (MM) agar was used with oleic acid (1 or 10 g/liter), lauric acid or hexane (both 1 g/liter), sodium acetate (50 mM), or a mixture of ethanol and glycerol (both 10 g/liter) as the carbon source.

**DNA procedures.** Plasmids and oligonucleotide primers used in this study are indicated in Tables S1 and S2, respectively, in the supplemental material. Standard recombinant DNA manipulations (22) were used throughout this study. PCRs were carried out by using high-fidelity polymerases (Roche). Cloned PCR fragments were sequenced to confirm the correct DNA sequence. For analyses of *P. chrysogenum* DNA and deduced amino acid sequences, the Clone Manager 5 program (Scientific and Educational Software, Durham, NC) was used.

For the visualization of peroxisomes by fluorescence microscopy, the peroxisomal targeting signal 1 (PTS1) Ser-Lys-Leu (SKL) was fused to the extreme C terminus of green fluorescent protein (GFP) and DsRed. For the construction of plasmid pGBRH2-GFP-SKL, a 0.74-kb SalI (blunted by Klenow treatment)-BamHI fragment containing the eGFP-SKL gene from plasmid pFEM36 was inserted between the BamHI and SmaI sites of *P. chrysogenum* expression vector pGBRH2. The related plasmid pBBK-001, carrying DsRed-....

**FIG. 1.** Schematic overview of the penicillin biosynthetic pathway. ACVS, 8-(L-α-aminoacidyl)-L-cysteinyl-β-valine synthetase; IPNS, isopenicillin N synthase; AT, acyl-CoA:6-amino penicillanic acid acyltransferase; PCL, phenylacetyl CoA ligase; PAA, phenylacetic acid.

**TABLE 1.** *P. chrysogenum* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL1951 (ATCC 9480)</td>
<td>Wild-type <em>P. chrysogenum</em></td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>NRRL1951 GFP-SKL</td>
<td>NRRL1951 expressing GFP-SKL under the control of P$_{gpdA}$</td>
<td>This study</td>
</tr>
<tr>
<td>Wis54-1255 (ATCC 28089)</td>
<td><em>P. chrysogenum</em> with improved PEN production</td>
<td>American Type Culture Collection</td>
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<tr>
<td>Wis54-1255 GFP-SKL</td>
<td>Wis54-1255 expressing GFP-SKL under the control of P$_{gpdA}$</td>
<td>This study</td>
</tr>
<tr>
<td>S2201</td>
<td>Non-oleate-utilizing mutant of Wis54-1255</td>
<td>This study</td>
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<tr>
<td>S2202</td>
<td>Non-oleate-utilizing mutant of Wis54-1255</td>
<td>This study</td>
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<tr>
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<td>Non-oleate-utilizing mutant of Wis54-1255</td>
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<tr>
<td>S2204</td>
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<td>DS17690</td>
<td>High-PEN-producing <em>P. chrysogenum</em> strain</td>
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<td>DS17690 GFP-SKL</td>
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<td>DS17690 ΔhdfA with increased homologous recombination</td>
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<tr>
<td>DS54465 GFP-SKL</td>
<td>DS54465 ΔniaD-P$<em>{gpdA}$-GFP-SKL-T$</em>{penDE}$</td>
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<td>DS17690 Pex3-GFP DsRed-SKL</td>
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<td>This study</td>
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<td>DsRed-SKL</td>
<td>DS17690 with DsRed-SKL under the control of P$_{gpdA}$</td>
<td>This study</td>
</tr>
<tr>
<td>Dnm1+++++</td>
<td>DsRed-SKL with integrated multiple copies of a P$<em>{pcbC}$-dnm1-His8-T$</em>{penDE}$ cassette</td>
<td>This study</td>
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</table>
SKL, was described previously (12). In gBRH2-GFP-SKL and pBBK-001, the GFP-SKL and DsRed-SKL genes are under the control of the strong P.chrysogenum pchb promoter. For lower-level, constitutive expression, we created derivatives in which the pchb promoter was replaced by the A. nidulans gpdA promoter. Primers BB-JK09 and BB-JK010 were used to amplify a 0.9-kb fragment comprising P_gpdA from pNiGANi, which was then digested with Asp718 and BamHI and cloned between the Asp718 and BamHI sites of both pBBK-001 and gBRH2-GFP-SKL, yielding pBBK-001 and pHWM-001, respectively. For the random integration of the P_gpdA and BamHI and cloned between the Asp718i and BamHI sites of both the Dnm1 protein was demonstrated by Western blotting using specific antib-

overexpression cassette were selected. Furthermore, the overproduction of 448-bp genomic fragment (data not shown). Strains with multiple copies of the selected for their ability to grow on acetamide as the sole nitrogen source. The resulting constructs were isolated as NotI fragments from pGBRH2-GFP-SKL and pHWM-001 and cotransformed into P. chrysogenum protoplasts with a 6.2-kb NotI-Spc1 fragment from plasmid pNiGANi comprising the A. nidulans amdS gene. Transformants were selected on acetamide plates, and green fluorescent colonies were selected for further use.

Multiple Gateway technology (Invitrogen) was used to construct a plasmid carrying a pex3-GFP fusion gene under the control of the A. nidulans gpdA promoter. First, the P. chrysogenum pex3 gene lacking a stop codon was isolated by PCR using primers attB1-f-pex3 and attB2-r-pex3-nostop using P. chrysogenum cDNA as a template (11). The resulting 1.8-kb fragment was then recombined into vector pDONR221, yielding pENTR221-pex3-nostop. Subsequently, this plasmid was recombined with plasmids pENTR41-P_gpdA, pENTR23-GFP-Tpex3, and pDST-R4-R3 to yield pEXP-PEpex3-GFP. Subsequently, a P. chrysogenum strain carrying both pex3-GFP and DsRed-SKL was obtained by cotransformation using DS7D90 protoplasts with a 6.0-kb Spel-NdeI fragment comprising the P_pex3-GFP-P_gpdA-Tpex3 cassette from pEXP-PEpex3-GFP. A 2.2-kb NotI fragment comprising the P_pex3-DsRed-SKL-Tpex3 cassette from pBBK-001 and the P_pex3-NdeI fragment from plasmid pNiGANi comprising the amdS cassette was transformed into acetamide plates. Subsequently, colonies displaying both green and red fluorescence were selected.

Construction of a P. chrysogenum dnm1 deletion strain. To enable site-specific gene deletion in P. chrysogenum, strain DS54465 was used (23). Since this strain cannot be provided with fluorescent markers by random integration, we constructed an nmd1-PtrpKS-GFP-SKL-Tpex3 cassette that enables the specific integration of the fluorescent marker in the P. chrysogenum nmd1 locus. First, we amplified the P_pex3-GFP-SKL-Tpex3 cassette with primers attB1-f-GpdA and attB2-r-3pex3 using pHWM-001 as a template. The resulting 2.2-kb fragment was recombined into pDONR221, yielding pENTR221-P_gpdA-GFP-SKL-Tpex3. Subsequently, this plasmid was recombined with pENTR41-PtrpKS, pENTR221-GFP, and pDST-R4-R3 to yield pEXP-5-Tspk-PtrpKS-GFP-SKL-Tpex3. This plasmid was linearized with NdeI and transformed into DS54465 protoplasts. NdeI-transformed colonies were selected on chlorate plates. A strain producing green fluorescent peroxisomes was designated DS54465 GFP-SKL and used for further study.

To delete the P. chrysogenum dnm1 gene, its promoter (1.0 kb) and terminator (1.2 kb) regions were PCR amplified by using primers BB-JK201 plus BB-JK202 and BB-JK203 plus BB-JK204, respectively, and recombined into Gateway vectors pIRE2-P_pex3 and pDST-R4-R3, resulting in plasmid pENTR241-DNM1-Prom and pENTR23-DNM1-Term, respectively. Finally, plasmids pENTR241-DNM1-Prom, pENTR221-AMDS, and pENTR23-DNM1-Term were recombined with the destination vector pDST-R4-R3, resulting in plasmid pEXP3-5-Dnm1-AMDS-3Dnm1. This plasmid was linearized with Sphi and transformed into P. chrysogenum DS54465 GFP-SKL protoplasts. Transformants were selected for their ability to grow on plates containing acetamide as the sole nitrogen source. The correct integration of the deletion cassette was tested via PCR using primers BB-JK05 and PgdA-See, resulting in a fragment of 1.1 kb (data not shown).

Construction of a P. chrysogenum dnm1 overexpression strain. In order to obtain dnm1 overexpression, the P. chrysogenum dnm1 gene was first amplified by PCR with primers BB-JK09 and BB-JK210 using P. chrysogenum cDNA (11) as a template, resulting in a 2.5-kb DNA fragment containing the entire dnm1 coding sequence lacking a stop codon. The PCR product was recombined into vector pDONR221, resulting in plasmid pENTR221-PdNm1. Subsequently, plasmids pENTR241-P_pex3, pENTR221-PcDNM1, and pENTR23-His8-Tpex3 were recombined into vector pDST-R4-R3/AMDS, resulting in expression vector pEXP-PdDNM1-His8. This plasmid was linearized with Sphi and transformed into P. chrysogenum DsRed-SKL protoplasts. Transformants were selected for their ability to grow on acetamide as the sole nitrogen source. The expression of the overexpression cassette was analyzed by PCR using primers BB-JK11 and BB-JK12, resulting in a 0.35-kb DNA fragment and a 448-bp genomic fragment (data not shown). Strains with multiple copies of the dnm1 overexpression cassette were selected. Furthermore, the overproduction of the Dnm1 protein was demonstrated by Western blotting using specific antib-

vides against the Saccharomyces cerevisiae Dnm1 protein or the His6 tag (data not shown).

Isolation of non-oleate-utilizing mutants. P. chrysogenum Wis54-1255 spores were mutagenized in potassium phosphate buffer (100 mM K2HPO4, 100 mM KH2PO4, pH 6.5) at 37°C using nitrocinoline oxide (1 mg/ml, final concentration). The incubation was terminated after 20 min by the addition of 2.5% KMnO4 or a mixture of glutaraldehyde (0.5%, vol/vol) and formaldehyde (2.5%, vol/vol) as described previously (33). Immunocytochemistry was performed on ultrathin sections of Unicryl-embedded samples by using antibodies raised against AT, GFP, or thiolase, as described previously (33).

Quantitative analysis of β-lactam levels. Quantitative H nuclear magnetic resonance (NMR) experiments were performed at 600 MHz on a Bruker Avance 600 spectrometer. A known quantity of internal standard (maleic acid) dissolved in phosphate buffer was added to a known quantity of filtrate prior to holfphilization. The residual was dissolved in D20 and measured at 300 K. The delay between scans (30 s) was more than five times the T1 of all compounds, so the ratio between the integrals of the compounds of interest and the integral of the internal standard is an exact measure for the quantity of the PEN samples.

Extracellular titers of PenG were determined by high-performance liquid chromatography (HPLC) using an isocratic flow of 310 g/liter acetonitrile, 640 g/liter acetic acid, 280 g/liter H2PO4, and 340 g/liter HPO4. Peaks were identified by comparison with a Shim-Pack XR-ODS 3.0-mm internal diameter by 75-mm column (Shimadzu) at a flow rate of 0.5 ml/min and detected at a wavelength of 254 nm. Intracellular β-lactam levels were determined by using mass spectrometry (5).

Fluorescence microscopy. Wide-field fluorescence imaging was performed by using a Zeiss Axios Observer Z1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Images were taken by using a Coolscope HQ2 camera (Roper Scientific Inc.). The GFP signal was visualized with a 470/40-nm-bandpass excitation filter, a 495-nm dichromatic mirror, and a 525/50-nm-bandpass emission filter. DsRed fluorescence was visualized with a 545/25-nm-band-pass excitation filter, a 570-nm dichromatic mirror, and a 605/70-nm-band-pass emission filter. z-stack images were made at an interval of 0.5 μm. Image analysis was carried out by using ImageJ (http://rsb.info.nih.gov/ij/image/).

Confocal laser scanning microscopy (CLSM) was performed by using a Zeiss LSM510 microscope (Zeiss Netherlands BV, Sliedrecht, Netherlands) equipped with a Zeiss Plan Apochromatic 63×1.4-numerical-aperture (NA) objective. GFP fluorescence was analyzed by the excitation of the cells with a 488-nm Ar/Kr laser with 1% output, and fluorescence was detected by using a BP 500-530 photo multiplier tube (PMT). DsRed fluorescence was analyzed by the excitation of the cells with a 543-nm He/Ne laser at 20%, and fluorescence was detected by using a 560-600-nm-long-pass filter. z-stack images were acquired with special software and 2D-reconstructed images were created by using ImageJ. The number of fluorescent spots was determined for grouped z-stack images of high-pitch cells. Statistical analysis was performed by using Student’s t-test.

For time-lapse recordings, the temperatures of the objective and subjective slide were kept at 25°C. Nine z-axis planes, each at 1-μm intervals, were acquired for each sample. The laser power (Ar-ion laser at 30 mW; 488 nm) was set to a 50% maximum value; the acousto-optical tunable filter (AOTF) was tuned down to 0.5%. Kymograms were created as described previously (34). ImageJ was used to create kymograms from time-lapse images. Slices were rotated to obtain a side view; subsequently, a z projection was created, yielding a kymogram.

RESULTS

Peroxisomes are required for efficient PEN biosynthesis. Earlier experiments suggested that peroxisomes are important for PEN production. To further elucidate the significance of peroxisomes in PEN biosynthesis, peroxisome-deficient mutants of P. chrysogenum strain Wis54-1255 were isolated essentially as described previously for baker’s yeast (3), by selecting non-oleate-utilizing strains. The survival rate of Wis54-1255 after mutagenesis with nitrocinoline oxide was determined at several time points (data not shown). Incubations of 20 min gave the best results, with a survival rate of 3%. As a first
screen for peroxisome deficiency, 5,614 surviving mutants were analyzed for their abilities to grow on oleic acid. False-positive mutants, due to a malfunctioning of the citric acid cycle or mitochondria, were weeded out on medium supplemented with acetate or ethanol-glycerol, respectively. Initially, six putative peroxisome-deficient \emph{P. chrysogenum} strains were isolated. Electron microscopy analysis showed greatly reduced peroxisomal profiles for all six mutants (data not shown). Additionally, PEN production levels of the mutant strains appeared to be 3 to 10 times lower than that of the Wis54-1255 control. Upon purification and conidiospore preparation, only four mutants produced stable, viable spore stocks. These mutants were designated S2201 to S2204. The mutants were unable to grow on medium supplemented with oleic acid as the sole carbon source. Also, growth on the fatty acid lauric acid or on hexane was fully inhibited (data not shown). To investigate the presence of intact peroxisomes, the mutants and the Wis54-1255 control were provided with a \emph{T}_{\text{gpdA-GFP-SKL}}-\emph{T}_{\text{penDE}} \text{ cassette, producing the peroxisomal marker protein GFP-SKL. After growth on PP medium, fluorescence microscopy analysis} (Fig. 2A) revealed that the GFP fluorescence was cytosolic in mutant strains S2201, S2202, and S2204, while S2203 cells show a partial mislocalization. (Left) Differential interference contrast (DIC) images. (Right) GFP fluorescence. (B and C) \(\beta\)-Lactam production by peroxisome-deficient mutants. The non-oleate-utilizing mutant strains S2201 to S2204 and the parental strain Wis54-1255 were cultivated on PP medium with POA, and PEN levels in spent media were determined by using NMR (B). Additionally, the intracellular amounts of the penicillin intermediates 6-APA (6-aminopenicillanic acid), IPN, and PEN were determined by mass spectrometry analysis (C). AU, arbitrary units. The error bars indicate the standard errors of the means (SEM).

**FIG. 2.** Analysis of peroxisome-deficient mutants. (A) Morphological phenotype of non-oleate-utilizing mutants. Shown is fluorescence microscopy analysis of cells of the non-oleate-utilizing mutants S2201 to S2204 and the parental strain Wis54-1255, all producing GFP-SKL, grown on PP medium. In the parental strain, the GFP fluorescence is present as distinct spots representing peroxisomes. In contrast, in cells of mutant strains S2201, S2202, and S2204, GFP-SKL is mislocalized to the cytosol, while S2203 cells show a partial mislocalization. (Left) Differential interference contrast (DIC) images. (Right) GFP fluorescence. (B and C) \(\beta\)-Lactam production by peroxisome-deficient mutants. The non-oleate-utilizing mutant strains S2201 to S2204 and the parental strain Wis54-1255 were cultivated on PP medium with POA, and PEN levels in spent media were determined by using NMR (B). Additionally, the intracellular amounts of the penicillin intermediates 6-APA (6-aminopenicillanic acid), IPN, and PEN were determined by mass spectrometry analysis (C). AU, arbitrary units. The error bars indicate the standard errors of the means (SEM).

A high-PEN-producing strain has elevated peroxisome numbers. To study the relationship between peroxisome num-
FIG. 3. Strains with enhanced PEN production contain increased peroxisome numbers. (A) Fluorescence microscopy images showing the peroxisome abundance in a hypha of a low-PEN-producing strain (NRRL1951) (top) and high-PEN-producing strain (DS17960) (bottom) grown under PEN-inducing conditions. Peroxisomes are marked by GFP-SKL. The pictures show overlays of bright-field and fluorescence images. Scale bars represent 10 μm. (B) Quantitative determination of peroxisomes marked by GFP-SKL in NRRL1951 and DS17690. Fluorescent spots were counted from grouped z stacks taken from randomly selected subapical cells by confocal laser scanning microscopy. (Left) Distribution pattern of peroxisomes in both strains. (Right) Average number of organelles per μm hypha. For each sample, at least 50 hyphae were counted. The error bars represent the SEM.

FIG. 4. Peroxisomes in P. chrysogenum contain both thiolase and AT. (A) P. chrysogenum Wis54-1255 was grown on PEN production medium in the presence (+) and absence (−) of oleic acid. Western blots of crude cell extracts with equal amounts of protein loaded per lane were decorated with anti-AT (α-AT) and anti-thiolase antibodies. The data show that AT is produced under both conditions, whereas thiolase is present predominantly in cells grown in the presence of oleic acid. (B) Detail of a section of a P. chrysogenum Wis54-1255 cell grown on PEN production medium supplemented with oleic acid. After immunolabeling using anti-AT (5-nm gold particles) and anti-thiolase (15-nm gold particles), both particles were observed on the microbody profile. N, nucleus; P, microbody; ER, endoplasmic reticulum. The bar represents 0.2 μm. (C) Peroxisome abundance is induced by oleic acid. P. chrysogenum DS17690 cells producing GFP-SKL were grown on PEN production medium in the presence (left) and absence (right) of oleic acid. The addition of oleic acid leads to an increase in peroxisome numbers relative to those of cells grown on induction medium alone. The arrow in the left panel indicates a cluster of peroxisomes. The top panels show fluorescence images, and the bottom panels show overlays of bright-field and fluorescence images.

Development of peroxisomes in PEN-producing P. chrysogenum strains. Previously, we demonstrated that P. chrysogenum contains true peroxisomes containing H$_2$O$_2$-producing oxidases and catalases (12). To investigate whether AT is targeted to these peroxisomes or to specialized organelles devoted solely to PEN biosynthesis (so-called “AT-somes” [18]), P. chrysogenum Wis54-1255 cells were grown on PP medium for 24 h to induce peroxisome proliferation, and oleic acid was subsequently added to the culture to simultaneously induce the synthesis of peroxisomal β-oxidation enzymes. During the adaptation of cells to oleic acid, the synthesis of one of the key enzymes of β-oxidation, thiolase, was indeed induced (Fig. 4A). Double-immunocytochemistry experiments using both anti-AT and anti-thiolase antibodies demonstrated that AT and thiolase were sorted to the same organelles (Fig. 4B). As observed for other fungi, the addition of oleic acid to PP medium resulted in a significantly enhanced number of peroxisomes per cell (Fig. 4C).

In the next series of experiments, we investigated the mode of peroxisome development in both germinating spores and developing hyphae. This was analyzed with the DS17690 GFP-SKL strain, as this strain contained the highest peroxisome numbers. Fresh conidiospores of this strain generally contained relatively few distinct GFP-SKL-containing spots, indic-
ative of peroxisomes. About 10% of the spores lacked such structures. The germination of peroxisome-lacking conidiospores was monitored by time-lapse CLSM. Generally, germination started after 10 h of incubation in production medium. Germination was preceded by a swelling of the spores, a phenomenon that was accompanied by the induction of GFP-SKL synthesis that was organized in reticular networks. Upon germination, presented in Video S1 in the supplemental material, the intensity of these structures increased and extended into the germination tube in which, however, they were rapidly observed as separate organelles. Stills from this video are presented in Fig. 5A. Similar networks have been observed to form in conidiospores that contained few peroxisomes (data not shown). To investigate the reticular networks in more detail, the peroxisomal membrane protein Pex3 was fused to GFP and coproduced with DsRed-SKL. The data (Fig. 5B and Video S2) show that in germinating spores, Pex3-GFP colocalized with DsRed-SKL, thus confirming the peroxisomal nature of these reticular networks and the distinct organelles developing from them (Fig. 5B). We interpret these data to mean that organelles present in the germination tube arise from the peroxisome reticular network in the conidiospore and are administrated to the growing hyphal tip. We have analyzed the site of peroxisome proliferation further by electron microscopy and analyzed serial sections cut through the neck between the germinating spore and germination tube. Using Amira software, we reconstructed a three-dimensional (3-D) model in which the peroxisomes are marked in red (see Fig. S1 in the supplemental material). This model supports the presence of interconnected peroxisome structures.

A remarkable feature of the growing hyphal tip is the continuous and virtually upward movement of organelles in the direction of the tip, including numerous GFP-SKL-containing peroxisomes (see Video S3 in the supplemental material). Kymogram analysis of the images demonstrated that the speed of tip growth exceeds the upward movement of the fluorescent spots (Video S3 and Fig. S2). This finding suggests that the main site of peroxisome development is in fact localized near the hyphal tips. Careful CLSM analysis indeed elucidated the presence of strongly fluorescent clusters of peroxisomes in these developing tips (Fig. 4C). The kymograms generated from CLSM images also demonstrated that in stain DS17960, cross wall formation in growing hyphae was not gradual during cell elongation but occurred simultaneously for several cross walls at the same time (Fig. S2). Frequently, several cross walls were formed at the same time, thereby partitioning the long hyphal tip in the corresponding compartments (Video S3 and Fig. S2). After septa had been formed, the upward movement of the fluorescent spots ceased (Fig. S2). The CLSM analysis also showed that the organelles incorporated into a subapical cell became strongly enhanced in fluorescence during prolonged cultivation as a result of the accumulation of the GFP-SKL protein (Fig. S2B). This finding suggests that these organelles are still competent to incorporate additional matrix components like newly synthesized PEN biosynthesis enzymes and, thus, further enhance PEN production rates in the corresponding cells in which they occur.

The peroxisome proliferation machinery in the hyphal tip requires the function of Dnm1. The dynamin-like protein Dnm1 is known to be essential for peroxisome fission in yeast and mammals (reviewed in reference 20). To determine whether this protein has a comparable function in organelle proliferation in hyphal tip cells, we have constructed P. chrysogenum strains lacking or overproducing Dnm1. P. chrysogenum GFP-SKL Δdnm1 cells and cells of the host GFP-SKL were grown for 48 h on PP medium and analyzed by CLSM. The data presented in Fig. 6A show that the peroxisomal structures in the tip of Δdnm1 cells have strongly expanded in conjunction with a reduction of the number of
peroxisomes in the rest of the cells. The presence of these clusters in the apical cells was even more pronounced in mutant cells grown in the presence of oleic acid (not shown). These data suggest that Dnm1 indeed has an important function in the formation of peroxisomes at the hyphal tip. Consistent with this was the observation that the number of peroxisomes in a dnm1 overexpression strain of *P. chrysogenum* was enhanced relative to those of wild-type (WT) controls (Fig. 6B).

To determine whether the absence or the presence of overproduced Dnm1 also affected antibiotic production, we determined PenG levels in spent media of the mutant strains and the WT host cultured on PP medium (Fig. 6C). Remarkably, the PEN production levels were slightly reduced in the dnm1 strain, while a major reduction in PenG levels was observed upon dnm1 overexpression. Similarly, we observed that growth on oleate, another condition that results in peroxisome proliferation, also results in decreased PEN production levels in the WT host.

**DISCUSSION**

In this paper we describe the correlation that exists between peroxisome numbers and PEN production rates in the filamentous fungus *P. chrysogenum*. First, we show that PEN production by *P. chrysogenum* requires the function of intact peroxisomes, as the synthesis of this secondary metabolite is significantly reduced in peroxisome-deficient mutants of *P. chrysogenum*. Most likely, peroxisomes create a unique microenvironment that is ideally suited to perform the two final steps of PEN production, namely, the activation of the side chain by PCL (14) and the substitution of the α-aminoacidipyl side chain for a hydrophobic one by AT (19, 35). For *A. nidulans*, a relationship between peroxisome function and PEN production was also observed previously (24). There, however, the correlation was less strict than that for *P. chrysogenum*, as peroxisome-deficient mutants of *A. nidulans* still showed high PEN titers (2). The present mutant studies and the significantly enhanced peroxisome numbers in high-producing strains like DS17960 demonstrate that intact organelles not only are crucial for efficient PEN production but also determine the ultimate production rates. However, it must be noted that not all our efforts to increase or decrease peroxisome numbers had the expected effect on β-lactam production. Although peroxisome numbers are highly upregulated by the addition of oleate to PP medium, we observed that PEN levels in spent medium were not increased but, rather, decreased (Fig. 6C). The reason for the decrease in PEN production levels may be the production of high levels of β-oxidation enzymes that compete with PEN production enzymes for peroxisomal CoA pools. Furthermore, the addition of oleate significantly affected the growth of *P. chrysogenum* cultures, which also results in reduced antibiotic production. A significant drop in PEN levels was also observed upon Dnm1 overproduction (Fig. 6C). We explain this surprising phenomenon by the effect that Dnm1 has on other organelles in this cell, i.e., its role in mitochondrial fission. Thus, our data imply that conditions that lead to peroxisome proliferation but simultaneously interfere with the normal physiology of the cell may be detrimental for antibiotic production. Previously, we demonstrated that the overproduction of Pex11, which exclusively affects peroxisomal profiles, led to an increase in PEN levels (13).

The second topic of our studies addressed the origin of peroxisomes in filamentous fungi. The analysis of germinating conidiospores demonstrated that the original peroxisome population following germination derived from a reticular network. As at the onset of germination, no distinct peroxisomes were detected in the spore, we cannot exclude that the initial organelle is formed via an alternative mode, e.g., from the endoplasmic reticulum (ER) (10, 17). However, upon spore swelling, the peroxisomal reticulum had already developed and most likely served as the source of the first organelles that migrated into the developing tip. During subsequent mycelial growth, peroxisome development occurred predominantly at the hyphal tip. Our data demonstrate that the fission of organelles from the reticular structure at the tip requires Dnm1. However, despite the absence of this dynamin-like protein (DLP), single peroxisomes could be observed in the apical and subapical cells. This finding suggests the formation of peroxisomes by alternative fission strategies (e.g., via the DLP Vps1, which is also conserved in filamentous fungi) or by the alternative formation of organelles from the ER. Recently, it was demonstrated that the fission of Woronin bodies, a special type of microbody required to close the septal pore upon hyphal...
damage, did not require the function of either Dnm1 or Vps1, although peroxisome numbers were apparently reduced in cells lacking dnm1 (16).

Remarkably, we found that septa in the P. chrysogenum strains under study are not formed successively, which is also the case for the NRR1:1951 type strain, but can develop at the same time. Following cross wall formation, we observed an increase in peroxisome size after prolonged cultivation due to the uptake of matrix proteins that continued in subapical cells. We assume that this leads to a gradual increase in the PEN production capacity of each individual cell as the cell matures. As yet, the intriguing question of what the advantage is for the organism of creating several subapical compartments at the same time is still unresolved. Possibly, this allows the organism to carefully partition organelle numbers over the separate compartments in order to form optimally adapted cells for performing various functions in, e.g., cell maintenance, energy generation, and metabolite production. Analyses that address these intriguing questions are ongoing.

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