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Expression of the transporter encoded by the *cefT* gene of *Acremonium chrysogenum* increases cephalosporin production in *Penicillium chrysogenum*

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**A B S T R A C T**

By introduction of the *cefEF* genes of *Acremonium chrysogenum* and the *cmeh* gene of *Streptomyces clavuligerus*, *Penicillium chrysogenum* can be reprogrammed to form adipoyl-7-amino-3-carbamoyloxyethyl-3-cephem-4-carboxylic acid (ad7-ACCCA), a carbamoylated derivative of adipoyl-7-aminoacetoxycephalosporanic acid. The *cefT* gene of *A. chrysogenum* encodes a cephalosporin C transporter that belongs to the Major Facilitator Superfamily. Introduction of *cefT* into an ad7-ACCCA-producing *P. chrysogenum* strain results in an almost 2-fold increase in cephalosporin production with a concomitant decrease in penicillin by-product formation. These data suggest that cephalosporin production by recombinant *P. chrysogenum* strains is limited by the ability of the fungus to secrete these compounds.

1. Introduction

Microbial resistance towards penicillins has shifted the demand for penicillins to cephalosporins because of their broad activity against Gram-positive and -negative bacteria (Weil et al., 1995; Barber et al., 2004). All medically useful cephalosporins are semisynthetically produced in processes in which 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA) are commonly used as the β-lactam nucleus for the various types of cephalosporins (Elander, 2003). These precursors are typically derived from a fermentation process that employs the filamentous fungus *Acremonium chrysogenum* as production host. However, due to a high demand for these precursors, in recent years research is focused on the development of a production process in *Penicillium chrysogenum* because of its high yields in antibiotics production. In 1995, Crawford et al. (Crawford et al., 1995; Schroen et al., 2000) heterologously expressed the *cefE* expandase gene of *Streptomyces clavuligerus* in *P. chrysogenum*. Upon addition of adipic acid to the growth medium, substantial levels of adipoyl-7-ADCA could be produced. Adipoyl-7-ADCA can be converted into 7-ADCA using enzymatic catalysis with glutarylacilase (Schroen et al., 2000) which is commonly used as precursor for semisynthetic cephalosporins. By a similar process, the combined *cefEF* expandase/hydroxylase of *A. chrysogenum* was expressed in *P. chrysogenum* yielding adipoyl-7-aminodeacetylcephalosporanic acid (adipoyl-7-ADAC). A co-integration with the *cefG* gene of *A. chrysogenum* encoding the DAC acyltransferase resulted in the conversion of adipoyl-7-ADAC into adipoyl-7-ACA (Crawford et al., 1995). Likewise, an in vitro conversion of adipoyl-7-ACA with a glutaryl-7-ACA-acylase yields 7-ACA (Monti et al., 2000) which is a preferred precursor for cephalosporin biosynthesis. Recently, the expression of the *A. chrysogenum* genes *cefD1*, *cefD2*, *cefE* and *cefC* in a *P. chrysogenum* strain lacking the isopenicillin N-acyltransferase has lead to significant amounts of intracellular produced deacetylcephalosporin C and cephalosporin C (Ullan et al., 2007). However, the exclusive intracellular localization suggests that *P. chrysogenum* lacks an efficient cephalosporin C secretion mechanism. Since adipoyl-7-ADCA, which has an adipoyl side chain, is secreted by *P. chrysogenum*, the (charged) amino group of the α-aminoadipate side chain of cephalosporin C might be an important barrier for secretion.

Several semi-synthetic cephalosporins contain a carbamoyl side chain at position 3, e.g. cefuroxime, cefoxitin and cefcapene pivoxil and these require adipoyl-7-amino-3-carbamoyloxyethyl-3-cephem-4-carboxylic acid (ad7-ACCCA) as the preferred precursor in semi-synthesis. This molecule cannot readily be produced from the 7-ACA or 7-ADCA precursors that can be produced by fermentation. Therefore, there is a high demand for a fermentative route that leads to the production of ad7-ACCCA. Production of ad7-ACCCA from adipoyl-6-APA can be achieved by introduction of three foreign enzymes into *P. chrysogenum* (Fig. 1) (Patent WO2004106347). Herein, adipoyl-6-APA is converted into adipoyl-7-ADCA by the combined expandase/hydroxylase CefEF of...
A. chrysogenum. Concomitant expression of the carbamoyl transferase gene cmcH of S. clavuligerus in this strain results in the carbamoylation of the 3’ hydroxyl position yielding ad7-ACCCA. Although a proof of principle of this fermentation process has been demonstrated (Patent WO2004106347), step optimization is required while it is also not clear how the cephalosporin derivatives are secreted (Patent WO2004106347), step optimization is required while it is also not clear how the cephalosporin derivatives are secreted (Patent WO2004106347). To obtain mycelium for transformation, spores were inoculated on YPG-medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) and grown for 40 h in a rotary incubator at 220 rpm and 25 °C. YPG-medium was also used to derive RNA from the cells. Ad7-ACCCA production was measured in a medium (g/l): Glucose, 5.0; Lactose, 80; Urea, 4.5; (NH4)2SO4, 1.1; Na2SO4, 2.9; KH2PO4, 5.2; K2HPO4, 5.2; K3HPO4, 4.8; terephthalic acid, 0.67; and adipic acid, 20.0; supplemented with spore solution (pH 6.5 ± 0.1).

2.2. Bacterial strain, cloning vectors

Escherichia coli DH5α was used as host strain for high frequency transformation, plasmid DNA amplification (Sambrook et al., 1989) and plasmid construction was performed with the MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) using the primer pairs indicated in Table 1. Vector pDONR® P4-P1R was used for cloning of the promoter region of 920 bp upstream of the pbcC gene. The attB4 site was added by polymerase chain reaction (PCR) using primer attB4-F-IPNS and the attB1R site was added with primer attB1-R-IPNS. The amplified PCR product was cloned into pDONR® P4-P1R using BP clonase creating pDONR™

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
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<tr>
<td>attB4-F-IPNS</td>
<td>GGGGACAACATTGTATGAAAAAGTGTCCTTA</td>
</tr>
<tr>
<td>attB1-R-IPNS</td>
<td>TACCTGGGCGTTTGTAGG</td>
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<tr>
<td>attB1F-cefT</td>
<td>CCGGACAACATTGTATGAAAAAGTGTCCTTA</td>
</tr>
<tr>
<td>attB2R-cefT-</td>
<td>TGGCGAACAATTGTATGAAAAAGTGTCCTTA</td>
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<tr>
<td>minus-Stop</td>
<td>GGGGACAACATTGTATGAAAAAGTGTCCTTA</td>
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<td>attB2F-His</td>
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<td>Tat</td>
<td>GCCCGGAAAAACCTTTCTGTACAAAAAAGCAGGCT</td>
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<tr>
<td>attB3R-GFP</td>
<td>TGGCGAACAATTGTATGAAAAAGTGTCCTTA</td>
</tr>
<tr>
<td>attB3R-Tat</td>
<td>GGGGACAACATTGTATGAAAAAGTGTCCTTA</td>
</tr>
</tbody>
</table>

Nucleotides in bold represent the att recombination sites of the Gateway® system, in italic represent nucleotides which maintain the frame between the HIS-tag, the GFP-tag and cefT gene. Underlined nucleotides represent the 8 amino acids of the HIS-tag.
P4-P1R-IPNS promoter. The pDONRTM 201 gateway vector was used to clone the cefT gene of *A. chrysogenum*. The attB1F site was added by PCR using primer attB1F-cefT and the attB2R site was added using attB2R-cefT-minus-Stop. The amplified cefT gene was cloned into pDONRTM 201 using BP clonase yielding pDONRTM 201-cefT. The stop codon of the cefT gene was removed to enable fusions with a His-tag or GFP-tag at the carboxyl-terminal site of the protein. To add the attB2F site to the terminator region of 551 bp downstream of the penDE gene for cloning in pDONRTM P2R-P3, primer attB2F-HisX8-Tat was used. Together with primer attB3R-Tat the terminator region was cloned with a His-tag and a stop codon was added to create vector pDONRTM P2-P3R-HIS-Tat. To obtain a carboxy-terminal fusion with GFP, primers attB2R-GFP and attB3R-Tat were used to create vector pDONRTM P2-P3R-GFP-Tat. Combining these pDONRTM vectors in the pDESTTM R4-R3 in the LR reaction of the Multisite GatewayTM Three-Fragment Vector Construction Kit a destination vector was made with respectively pcbC promoter, cefT gene, His- or GFP-tag and the terminator of the penDE gene. All plasmids used in this study are summarized in Table 2.

### 2.3. Transformation to *P. chrysogenum*

Protoplasts were isolated and transformation (Alvarez et al., 1987) was done by co-transformation of the pDESTTM P4-R3 vector with pcbC promoter, cefT gene and penDE terminator and the ace-amidase-gene (*amdS*) used as selection marker on plates with acetic acid as sole nitrogen source (Kolar et al., 1988).

### 2.4. Southern blotting and hybridizations

Genomic DNA of *P. chrysogenum* DS17690, the ad7-ACCCA producing strain DS49834 and derived transformants was isolated using the E.Z.N.A. Fungal DNA kit (Omega Bio-tek). Genomic DNA (2.5 μg) was digested using the appropriate restriction enzymes, separated on a 0.8% agarose gel and blotted onto a Zeta-Probe membrane (Biorad) (Sambrook et al., 1989). Probes were labeled with digoxigenin using the PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer’s instructions. Hybridizations were done overnight at 42 °C in hybridization buffer (50% formamide, 5× SSC, 2% blocking reagent [Roche], 0.1% Na-lauroylsarcosine, 0.02% SDS) and the membranes were washed twice with 2× SSC, 0.1% SDS for 15 min and twice with 0.2× SSC, 0.1% SDS. Digoxigenin-labeled probes were detected by chemiluminescence using CPD-star (Roche).

### 2.5. Reverse transcriptase PCR

Total RNA of transformants was isolated using Trizol® (Invitrogen) and positive transformants were determined with RT-PCR beads (Amersham) using a cefT specific primer (cefT F1: 5′-TGATTCCATGACAGACGCAGCC-3′) and a attB2 - HIS-tag primer (5′-TGTTGATGTGATGGTGGACACCTTT-3′) to obtain a RT-PCR fragment of 384 bp. For the semi-quantitative RT-PCR on the heterologously expressed genes and γ-actin as control, the primers in Table 3 were used to amplify mRNA fragments with RT-PCR beads. Primers for γ-actin and cefT were developed that flank both sides of an intron present in these genes. These ensured that RT-PCR fragments were amplified from mRNA instead of genomic DNA.

### 2.6. NMR analysis

Quantitative 1H NMR experiments were performed at 600 MHz on a Bruker Avance 600 spectrometer. To a known quantity of filtrate, an internal standard (malic acid) dissolved in phosphate buffer was added prior to lyophilisation. The material was dissolved in D$_2$O and analyzed by NMR at 300 K. The delay between scans (30 s) was more than five times T$_1$ 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 penicillins and cephalosporins.

### 2.7. Confocal fluorescence microscopy

Confocal images were made using a Zeiss LSM510 confocal laser scanning microscope (CLSM; Zeiss Netherlands, Weesp, The Netherlands). GFP fluorescence was analyzed by excitation the mycelium with a 488-nm argon ion laser and detection of fluorescence emission using a band pass (BP) 500–550 nm filter. For vacuolar stains with the FM4-64 dye, mycelium were grown for 48 h in YPG medium and samples were stained with 2 μM FM4-64 for 30 min, washed in fresh YPG medium and incubated for 90 min. Fluorescent microscopy of the FM4-64 stained cells was performed with a fluorescence microscope (Axioskop, Zeiss) using appropriate filter sets for the GFP and FM4-64 fluorescence. Images were captured with a AxioCam camera (Zeiss).

### 3. Results

#### 3.1. Integration of the cefT gene into the genome of *P. chrysogenum*

By introduction of the cefEF genes of *A. chrysogenum* and the *cmcl* gene of *S. clavuligerus*, *P. chrysogenum* can be reprogrammed to form adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (ad7-ACCCA), a carbamoylated derivate of 7-ADCA (Patent WO2004106347) (Fig. 1). To access the functional impact of the cephalosporin transporter CefT of *A. chrysogenum* on the production of ad7-ACCCA, the cefT gene was amplified by PCR from chromosomal DNA derived from *A. chrysogenum* and cloned into pDESTTM R4-R3 expression vector (Invitrogen). A HIS-tag was introduced at the carboxyl terminus and the cefT gene was placed under the control of the penDE promoter. By introduction of the cefT gene as assessed by RT-PCR of total RNA derived from mycelium grown for 48 h on YPG. Two out of the transformants (cefT1 and cefT2) were selected for further analysis as these

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**Table 2**

<table>
<thead>
<tr>
<th>Cloning Gateway&lt;sup&gt;®&lt;/sup&gt; cloning</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>pDONRTM P4-P1R-IPNS promoter</td>
<td>pcbC promoter</td>
</tr>
<tr>
<td>pDONRTM R4-P2R-HIS-Tat</td>
<td>cefT gene of <em>A. chrysogenum</em></td>
</tr>
<tr>
<td>pDONRTM P2-P3R-GFP-Tat</td>
<td>GFP-tag with C-terminal terminator of penDE gene</td>
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<tr>
<td>pDest P4-R3 AcCefT-HIS</td>
<td>Expression plasmid cefT gene of <em>A. chrysogenum</em> fused to His-tag</td>
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<tr>
<td>pDest R4-R3 AcCefT-GFP</td>
<td>Expression plasmid cefT gene of <em>A. chrysogenum</em> fused to GFP-tag</td>
</tr>
<tr>
<td>pBlueScript-AMDS</td>
<td>Co-transformation plasmid with <em>amdS</em> gene</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′) [For/Rev]</th>
<th>Fragment (bp)</th>
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<tr>
<td>actin</td>
<td>CGGCGGAGTATCCAGCTACGCTGCAGGCTTGGATGCCGACCTCC</td>
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<td>cefEF</td>
<td>GTCAAGGCGGAGCAGACGGGAGCACTCAGAGACAGACAG</td>
<td>259</td>
</tr>
<tr>
<td>cmcl</td>
<td>GGTACGACGTCGACATTACACGATGCTTCATGCTTCAC</td>
<td>229</td>
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</tbody>
</table>
showed increased levels of ad7-ACCCA production as assessed by NMR (see below). Some of the transformants had completely lost the ability to produce ad7-ACCCA which in all cases could be attributed to a loss of the cefT gene (data not shown). In independent transformations, an additional three transformants were obtained with increased levels of ad7-ACCCA production. The chromosomal integration of the cefT gene and the presence of the cefEF and cmcH genes was confirmed by Southern analysis (Fig. 2). Genomic DNA of the two cefT transformants, the wild-type strain DS17690 and parental ad7-ACCCA producing strain DS49834 was digested with the NdeI, Ndel or BamHI, which recognize single restriction sites in the cefEF, cmcH and cefT genes, respectively. Depending on the number, size and intensity of restriction fragments, this allows for the detection of single and multiple genomic integrations of the analyzed genes including insights in their orientation. To detect the cefEF gene in the various strains, a 978 bp (97.8% of the gene) fragment of the cefEF gene was used as a probe. With Ndel, a head-to-tail orientation of integrated genes would yield a 3352 bp band, while a head-to-head orientation should yield a 2507 bp fragment. As expected cefEF was absent in the wild-type strain DS17690, but appears to be present as two copies organized in a head-to-tail orientation and as an individual third copy in the DS49834 ad7-ACCCA producing strain (Fig. 2 A). These integrations are also present in the two cefT transformants that were selected for further analysis. For the cmcH gene, a 1479 bp (94.4% of the gene) fragment was used as a probe. Southern analysis suggests the presence of four copies of the cmcH gene in the DS49834 ad7-ACCCA producing strain (Fig. 2B), while in the cefT transformants, only three of these four copies could be detected. Unlike the cefEF integrations which are clustered, cmcH seems to integrate into multiple loci. For the cefT gene (Fig. 2 C), a 397 bp (22.7% of 3’-end of the cefT gene) fragment was used as a probe, and a head-to-head orientation of integrated genes should yield a 5222 bp fragment. Both transformants appear to contain three copies of the cefT gene, two copies arranged in a head-to-tail orientation and one additional copy. The Southern blots, however, also show the genomic instability of the DS49834 ad7-ACCCA strain used in this study since the transformation protocol resulted in a loss of copies of the cmcH gene but this did not result in a loss in the ability to produce ad7-ACCCA. On the other hand, the transformants that had lost the ability to produce ad7-ACCCA also has lost the cefEF gene (data not shown). Importantly, the Southern blotting demonstrates the successful integration of the cefT gene into the two selected DS49834 ad7-ACCCA producing strains.

3.2. Transcription of cefT, cefEF and cmcH genes

The transcription of the heterologous expressed genes cefT, cmcH and cefEF in P. chrysogenum was analysed after growth in shake flasks using ad7-ACCCA producing medium containing adipic acid. Total RNA was isolated using Trizol after 7 days of growth. Semi-quantitative RT-PCR was used to determine the transcription levels and the optimal, non-saturating number of PCR cycles was determined for cefT, cefEF, cmcH and the internal standard actin at respectively 16, 19, 20 and 19 cycles. Expression of the cefT gene is approximately comparable in the two transformants, and no expression was observed in the DS17690 wild-type strain and the parental ad7-ACCCA producing strain DS49834. CefEF was expressed at similar levels in the two transformants and the parental DS49834 ad7-ACCCA producing strain (Fig. 3). Expression of the cmcH gene appeared more variable in the transformants as compared to the parental strain which is likely related to the loss of at least one copy in the cefT transformants. As expected no transcripts of cefT, cefEF and cmcH were observed in the wild-type strain DS17690.

3.3. Cellular localization of CefT

To further validate the expression of cefT gene and the cellular localization of the gene product, the cefT gene was fused at its 3’-end with the gfp gene. The fusion construct was cloned into the pDEST™ R4-R3 expression vector and placed under control of the pbcC promoter and the transcriptional terminator of penDE. P. chrysogenum DS17690 strain was co-transformed with plasmids pDEST™ R4-R3 AcCefT-GFP and pBlueScript-AMDS, the latter of which contains the acetamidase-gene, amdS. Transformants were selected by growth on acetamide as sole nitrogen source. AmdS positive transformants were transferred into YPG-medium and selected for cefT-gfp expression by fluorescence microscopy. Although the pbcC promoter is repressed by glucose (Martin et al., 1999) the activity of this promoter under such growth conditions is still sufficiently high for fluorescent analysis. For localization, conidia were incubated for 24 and 48 h in YPG whereupon the fungal cells are analyzed by fluorescent microscopy. This revealed a bright fluorescence staining of plasma membrane (Fig. 4A–C). However, already after 24 h some of the fluorescence was found to localize in smaller organelles, that in separate experiments using the vacular membrane dye FM4-64 were assigned as vacuoles (see below). This suggests the proteolytic degradation of the CefT-GFP fusion leaving the stable GFP moiety in the vacuoles.
Even after 48 h, CefT-GFP could be localized at the plasma membrane, but under those conditions, GFP localizes mainly to the vacuoles (Fig. 4D–G). Western blot analysis using an antibody directed against GFP confirmed the massive degradation of CefT-GFP showing high levels of free GFP (data not shown). These data demonstrate that CefT correctly localizes to the plasma membrane but at later stages also substantial targeting of the protein occurs to the vacuoles for degradation.

3.4. Extracellular production of ad7-ACCCA

The production of ad7-ACCCA by the cefT transformants and the parental DS49834 ad7-ACCCA strains was determined after 7 days of growth in ad7-ACCCA producing medium containing adipic acid as the side chain precursor. Mycelium was removed by centrifugation and the supernatant was isolated and analyzed by NMR. As compared to the parental DS48934 strain, the ad7-ACCCA production by the transformants expressing the cefT gene increased by 1.7-fold (Fig. 5A). Interestingly, also the ad7-ACCCA precursor ad7-AHCA, a cephem precursor with a 6-membered ring, increased with 1.3–1.4-fold, while 5-membered penam precursors such as isopenicillin N and adipoyl-6-APA levels were decreased. Production was further analysed in time, and compared for the DS48934 strain with and without cefT expression (Fig. 5B). The presence of CefT results in a substantial improvement of ad7-ACCCA production which peaks up to 2.4-fold after 5–7 days as compared to the parental strain. These data suggest that the introduction of a cephalosporin transport system into \textit{P. chrysogenum} results in a significant improvement of the production of ad7-ACCCA with the concomitant reduction of 5-membered penam by-products.

4. Discussion

Cephalosporin is the largest antibiotic market to date and semisynthetic cephalosporin production depends about one third on the use of 7-ADCA as precursor and about two thirds proceeds via 7-ADCA (Elander, 2003). The chemical processes that yield 7-ADCA and 7-ACA derived antibiotics are precursors which can be easily converted in down-streaming processes into cefuroxime, cefoxitin and cepapenepivoxil can be worthwhile. A novel biosynthetic pathway towards the production of the carbamoylated derivative of adipoyl-7-ADAC, ad7-ACCCA, is based on the introduction of the \textit{A. chrysogenum} CefEF dual function enzyme with expandase and hydroxylase activity and the \textit{S. clavuligerus} CmcH enzyme with carbamoyltransferase activity into a penicillin production \textit{P. chrysogenum} strain (Patent WO2004106347). Cephalosporins are normally not produced by \textit{P. chrysogenum} and thus represent foreign molecules to these cells. This implies that both intracellular and plasma membrane transport steps could be potential bottlenecks in a complete fermentative production process. CefT of \textit{A. chrysogenum} encodes a transporter of the Major Facilitator Superfamily that has been implicated in the secretion of cephalosporin C. Therefore, we have introduced the cefT gene of \textit{A. chrysogenum} into the ad7-ACCCA-producing \textit{P. chrysogenum} DS17690 strain. Most of the transformants obtained showed a poorer production of ad7-ACCCA, and Southern blotting in all of these strains revealed the loss of cefT copies, suggesting a genomic instability of this strain. Also the cefT mRNA levels were decreased in these transformants. The instability likely emerges from the protoplasting and possibly the random integration of the cefT and cmcH gene in the genome. To date no heterologous recombination hotspots in \textit{P. chrysogenum} are known but heterologous recombination hotspots might explain the instability of the strain used in our experiments. In \textit{Saccharomyces cerevisiae}, however, meiotic recombination hotspots have been investigated which have preferred targeting for heterologous recombination (Lichten and Goldman, 1995).

Importantly, also cefT transformants were obtained that showed increased levels of ad7-ACCCA production. It should be stressed that in the absence of cefT, the transformation procedure did not result in transformants with increased levels of ad7-ACCCA production. Transcriptional analysis indicates that in the improved strains, cefT is expressed in conjunction with cefEF and cmcH. Likewise, cellular localization of CefT as a fusion protein with GFP suggests an initial localization to the plasma membrane, but after sustained growth, free GFP accumulates in the vacuolar lumen. The latter is likely due to the proteolytic degradation of the CefT-GFP fusion protein which is either caused by the presence of the GFP marker, the high expression driven by the strong pbC promoter and/or the heterologous origin of CefT. Nevertheless, even after longer periods of growth, a substantial level of GFP fluorescence could be detected at the plasma membrane suggesting the correct sorting and localization of the CefT transporter. The NMR analysis indicates a more than 2-fold increase in the levels of extracellular ad7-ACCCA concentration upon the expression of cefT in the DS49834 ad7-ACCCA producing strain. Also, the concentration of the by-product ad7-AHCA increased, while the amounts of 5-membered penam precursors like IPN and ad6APA decreased. This suggests that in the presence of CefT, a larger fraction of the 5-membered penam precursors are converted by CefEF. The most plausible explanation of this phenomenon is that in the absence of CefT, cephalosporin secretion is a limiting step in the biosynthetic cascade resulting in the intracellular accumulation of cephalosporin derivatives that inhibit the expandase activity of CefEF. Consequently, the through-put in the pathway is slowed down and 5-membered penam precursors start to accumulate or escape CefEF whereupon they are excreted by endogenous transport systems. The increased production of cephalosporins is therefore likely the consequence of a more rapid excretion of these antibiotics, i.e., both ad7-ACCCA and ad7-AHCA thereby preventing feedback inhibition of the biosynthetic enzymes in the pathway. These data also suggest that CefT is specific for cephalosporins and either is inactive or poorly active with the 5-membered penam com-
pounds. In *A. chrysogenum*, CefT is involved in the excretion of cephalosporin C (Ullan et al., 2007) which in contrast to ad7-ACCCA contains the positively charged amine moiety of α-aminoadipate as side chain. Interestingly, there is no secretion after the formation of cephalosporin C in *P. chrysogenum* (Ullan et al., 2007) which would imply that this fungus does not contain endogenous transporters that can handle this molecule. In contrast, ad7-ACCCA and ad7-AHCA are secreted by *P. chrysogenum* indicating that a charged side chain prevents the secretion of cephalosporins by the endogenous transport systems. In particular for such derivatives, the presence of CefT will likely promote secretion and production. The genome of *P. chrysogenum* contains at least 150 potential CefT homologs with similarities ranging from 37–84% (van den Berg et al., submitted for publication). However, it should be stressed that with MFS transporters, amino acid sequence similarity is a poor indicator for substrate specificity. Thus these systems might be involved in a variety of unrelated uptake and excretion processes. We are currently analyzing the role of these systems in secondary metabolite formation by means of transcriptional analysis, protein overexpression and gene knock-outs.

**Fig. 4.** Membrane localization of CefT expressed in *P. chrysogenum* DS17690 cefT1 after 24 (A–C) and 48 (D–G) hours of growth in YPG. (A and D) Fluorescence image showing CefT-GFP localization in the plasma membrane, and after longer periods of growth, also in vacuoles. (B) Bright field image. (E) Fluorescence image showing FM4-64 localization in the vacuolar membrane. (C, F and G) Merged image.
Acknowledgments

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References


