The transporter CefM involved in translocation of biosynthetic intermediates is essential for cephalosporin production

Fernando TEIJEIRA*, Ricardo V. ULLÁN†, Susana M. GUERRA*, Carlos GARCÍA-ESTRADA†, Inmaculada VACA† and Juan F. MARTÍN*†

*Área de Microbiología, Departamento de Biología Molecular, Facultad de CC. Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n. 24071, León, Spain, and †Instituto de Biotecnología de León (INBIOTEC), Parque Científico de León, Av. Real, 1, 24006, León, Spain

The cluster of early cephalosporin biosynthesis genes (pcbAB, pcbC, cefD1, cefD2 and cefT of Acremonium chrysogenum) contains all of the genes required for the biosynthesis of the cephalosporin biosynthetic pathway intermediate penicillin N. Downstream of the cefD1 gene, there is an unassigned open reading frame named cefM encoding a protein of the MFS (major facilitator superfamily) with 12 transmembrane domains, different from the previously reported cefT. Targeted inactivation of cefM by gene replacement showed that it is essential for cephalosporin biosynthesis. The disrupted mutant accumulates a significant amount of penicillin N, is unable to synthesize deacetoxy-, deacetyl-cephalosporin C and cephalosporin C and shows impaired differentiation into arthrospores. Complementation of the disrupted mutant with the cefM gene restored the intracellular penicillin N concentration to normal levels and allowed synthesis and secretion of the cephalosporin intermediates and cephalosporin C. A fused cefM-gfp gene complemented the cefM-disrupted mutant, and the CefM-GFP (green fluorescent protein) fusion was targeted to intracellular microbodies that were abundant after 72 h of culture in the differentiating hyphae and in the arthrospore chains, coinciding with the phase of intense cephalosporin biosynthesis. Since the dual-component enzyme system CefD1–CefD2 that converts isopenicillin N into penicillin N contains peroxisomal targeting sequences, it is probable that the epimerization step takes place in the peroxisome matrix. The CefM protein seems to be involved in the translocation of penicillin N from the peroxisome (or peroxisome-like microbodies) lumen to the cytosol, where it is converted into cephalosporin C.

Key words: Acremonium chrysogenum, CefM transporter, cephalosporin C biosynthesis, compartmentalization, isopenicillin N, translocation of penicillin N from microbodies.

INTRODUCTION

Industrial production of cephalosporins is performed with the filamentous fungus Acremonium chrysogenum [1,2], although the ability to produce cephalosporin is also present in other fungi [3], including Kallichromatia tethys, a marine fungus in which some genes for cephalosporin biosynthesis have been reported previously [3a].

The genes involved in CPC (cephalosporin C) biosynthesis in A. chrysogenum are arranged in two separate clusters: (i) the ‘early’ cephalosporin cluster that includes the genes involved in the four initial steps of the biosynthetic pathway [pcbAB encoding ACV (δ-L-α-aminoadipyl-L-cysteinyl-D-valine) synthetase, pcbC encoding IPN (isopenicillin N) synthase, cefD1 encoding isopenicillinyl N-CoA synthetase, and cefD2 encoding isopenicillinyl N-CoA epimerase] located in chromosome VII (4.6 Mb), and (ii) a ‘late’ cephalosporin cluster [cefEF encoding DAOC (deacetoxycephalosporin C) synthase (expandase)/DAC (deacetylcephalosporin C) hydroxylase, cefG encoding acetyl-CoA:DAC acetyltransferase] in chromosome I (2.2 Mb) [4–7].

This arrangement of the cephalosporin genes in two separate clusters is intriguing and contrasts with the organization in Penicillium chrysogenum where all of the genes (pcbAB, pcbC and penDE) of the penicillin pathway are clustered in a single 17 kb DNA region [8] located in chromosome I (10.4 Mb) [9]. These observations suggest that the cephalosporin pathway has been evolutively assembled from two different parts encoded by separate DNA fragments [10].

In Penicillium chrysogenum, some specific steps of the β-lactam biosynthesis pathway are compartmentalized within fungal cells; the first two steps (formation of the ACV tripeptide and its cyclization to IPN) occur in the cytosol [11,12], whereas the last steps (activation of phenylacetic acid to phenylacetyl-CoA and the conversion of IPN into benzylpenicillin) occur in peroxisomes [13,14].

In A. chrysogenum, it was initially believed that all steps have a cytosolic location [11,12]. However, recent evidence suggests that the epimerization of IPN to PenN (penicillin N) is catalysed by peroxisomal enzymes (R.V. Ullán, I. Vaca, F. Teijeira and J.F. Martín, unpublished work). This compartmentalization requires specific membrane transport steps across the peroxisomal membranes. Nothing is known about peroxisomal transporters in these fungi. The cefT [15] and atrD [16] -encoded transporters appear to be related to secretion of β-lactam antibiotics out of the cells. In A. chrysogenum, the previously reported cefT gene [15] is located in the early cephalosporin cluster (chromosome VII)

Abbreviations used: ACV, δ-L-α-aminoadipyl-L-cysteinyl-D-valine; CCM, complex culture medium; CPC, cephalosporin C; DAC, deacetylcephalosporin C; DAOC, deacetoxycephalosporin C; DP, defined production; DTT, dithiothreitol; GFP, green fluorescent protein; IPN, isopenicillin N; LPE, Le Page and Campbell; MFS, major facilitator superfamily; ORF, open reading frame; PenN, penicillin N; Pex19, peroxisome biogenesis factor 19; PTS, peroxisomal targeting signal; RT, reverse transcription; TCM, transformant complemented in cefM; TDM, transformant disrupted in cefM; TMS, transmembrane segment.

1 To whom correspondence should be addressed (email jf.martin@unileon.es).

The nucleotide sequence data reported for cefM has been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AM231815.
downstream of pchAB and encodes a multidrug efflux pump protein belonging to the MFS (major facilitator superfamily) of membrane proteins. The CefT protein confers resistance to some toxic organic acids including isovaleric acid and phenylacetic acid. Overexpression of the cefT gene in A. chrysogenum resulted in a 2-fold increase in CPC production. Nevertheless, targeted inactivation of cefT with the double-marker technique [17] revealed that cefT is not essential for cephalosporin biosynthesis [7,15] and suggested the presence of redundant systems involved in cephalosporin secretion.

In order to search for proteins involved in cephalosporin secretion, we have characterized the genes in the region located downstream of the cefD1 gene (distal to cefT) in the early cephalosporin gene cluster of A. chrysogenum. In the present paper, we report the characterization of a new gene, cefM, that encodes a membrane protein belonging to the MFS and its essential role in β-lactam transport.

The microbodies are single membrane-bound organelles that contribute to the α- and β-oxidation of fatty acids, bile acids, cholesterol and several other toxic compounds. A class of microbodies has been identified as peroxisomes [18]. The mechanism of import of peroxisomal matrix proteins has been partially elucidated (reviewed in [19]). There are several types of PTS (peroxisomal targeting sequence): for matrix proteins (PTS-1 and PTS-2) [19] and for membrane proteins [20,21]. These targeting sequences for peroxisomal membrane proteins contain a basic amino acid combined with at least one transmembrane region [22–24]. These proteins are recruited to the cytoplasm by Pex19 (peroxisome biogenesis factor 19) [25], and the consensus sequences for binding to Pex19 have been identified [26].

It was therefore of interest to study whether the MDR (multidrug resistance) transmembrane proteins encoded by genes located in the ‘early’ cephalosporin cluster are targeted to the peroxisomal membrane. We describe, for the first time, the compartmentalization in microbodies of one part of the CPC biosynthesis pathway.

**EXPERIMENTAL**

**Microorganisms and culture media**

*Acremonium chrysogenum* C10 (ATCC 48272), a high-cephalosporin-producing strain available from PanLabs, was used in the present study. For sporulation, *A. chrysogenum* C10 and the mutant strains were grown in solid LPE (Le Page and Campbell) medium [7,15] for 7 days at 28°C. Spores and mycelium fragments collected from six plates of LPE medium were suspended in 100 ml of LPE medium and incubated at 30°C for 48 h in an orbital incubator at 250 rev./min. Then, 10 ml of seed culture was used to inoculate 100 ml of Shen’s DP (defined production) medium. The cultures were incubated in 500 ml triple-baffled flasks (Bellco) containing 100 ml of medium at 25°C in a rotary shaker at 250 rev./min. Samples were taken every 24 h, and cephalosporin production was determined by bioassays against *Escherichia coli* ESS2321 as the test strain in plates with penicillinase (from *Bacillus cereus* UL1) as described previously [27]. For confocal laser-scanning microscopy studies, the *A. chrysogenum* strains were grown in CCM (complex culture medium) [27a].

**Library screening**

A gene library of *A. chrysogenum* C10 was constructed in the ble-EMBL3 vector as described previously [4]. The *E. coli* LE392 strain was infected with the phages contained in the gene library of *A. chrysogenum* C10. The DNA of phages in the lysis plaques of the infection plates was transferred on to Hybond-NN membranes (GE Healthcare). In order to find the positive phages with the new ORF (open reading frame), two or three consecutive rounds of hybridizations were carried out using the appropriate digoxigenin-labelled probe according the DIG Easy Hyb system (Roche Diagnostics) as described by Ullán et al. [15]. Finally, the DNA fragment containing the cefM gene was cloned and sequenced.

**DNA isolation and Southern blotting**

Genomic DNA of *A. chrysogenum* was isolated as described previously [7]. Samples of genomic DNAs (3 μg) from *A. chrysogenum* C10 and its transformants were digested with restriction enzymes and separated using 0.7 % agarose gels. The gels were blotted on to Hybond-NN membranes as described by Ullán et al. [15]. For Southern blot analysis, the DIG Easy Hyb system was used. Hybridizations were performed according to the manufacturer’s protocol, and the hybridization signals were visualized by chemiluminescence and recorded on X-ray film with an exposure time of 5 min.

**Plasmids containing the cefM gene**

**pcefMa and pcefMb**

The EcoRV fragment of 3.1 kb containing the cefM gene under the control of its own promoter was placed in two opposite orientations (α or β) at the unique EcoRV site of pZErO-2 (Invitrogen).

**pDM**

This plasmid contains a disrupted cefM gene that was obtained by insertion of the bleomycin resistance (*ble*) cassette (1.47 kb fragment) from pJL43 [4] into the XhoI site. The hygromycin resistance (*hph*) cassette, subcloned from pAN7-1 [17], was inserted in the BamHI site.

**pCM**

This plasmid was used for trans complementation of the cefM disruptant strain. To construct pCM a HindIII/XbaI 3.2 kb fragment from pcefMa was inserted into the HindIII/XbaI site of pAN7.1.

**pcefM-gfp**

To obtain the fused cefM-gfp gene, an Ecl136I 2.6 kb DNA fragment obtained from plasmid pcefMa by PCR using the M13 Forward and MC (5’-GAGCTCCCCACAGCGCTGGCTGAA-3’) oligonucleotides was inserted into the EcoRI site of pMCBI5 bearing the gfp2.5 gene of *Aequorea victoria* encoding GFP (green fluorescent protein) [28].

**DNA sequencing**

Sequencing reactions of the DNA were carried out using standard procedures [15], and automatic sequencing was performed with the AutoRead™ System (GE Healthcare).

**RNA extraction and intron analysis**

Total *A. chrysogenum* RNA was isolated with the RNeasy kit (Qiagen) as described previously [29]. To elucidate the presence of putative introns in the DNA sequence of the cefM gene (ORF10), the DNA region containing the expected intron-splicing sites was
amplified by RT (reverse transcription)–PCR (Promega) using RNA of *A. chrysogenum* 48 h cultures as template with the primer pairs I1-R (5′-GGCGGGAGGTGGGACCTGA-3′) and I1-F (5′-TGAGATGGCGCG-GGATGAAAGAC-3′) as substrates. The reaction signal was detected using NBT (Nitro Blue Tetrazolium) and BCIP (5-bromo-4-chloroindol-3-yl phosphate) (Roche Diagnostic) as substrates.

A. chrysogenum transformation

Transformation of *A. chrysogenum* protoplasts was performed as described previously [4]. Transformants were selected in TSA (tryptic soy agar) (Difco) supplemented with sucrose (0.30 M; 10.3 %) as osmotic stabilizer and phleomycin (10 μg/ml) or hygromycin B (30 μg/ml) as selection markers.

HPLC determination of IPN, PenN, DAC and CPC

The extracellular and intracellular concentrations of IPN, PenN, DAC and CPC of *A. chrysogenum* strains were determined in a Waters HPLC System equipped with a 2487 dual-absorbance detector, using a 4.6 mm × 250 mm RPC18 LichroSpher® 100 column (Merck). The elution system used was as described previously by Ullán et al. [7,27].

Cell extracts and Western blotting

Mycelia from the different *A. chrysogenum* strains were centrifuged at 4400 g for 10 min and washed three times in 0.9 % NaCl. Then, they were resuspended in cold TD buffer [50 mM Tris/HCl (pH 8.0), 5 mM DTT (dithiothreitol) and Complete™ solution for protease inhibition (Roche Applied Science)]. Disruption of cells was achieved by sonication on ice using six pulses of 20 s with 60 s intervals between each pulse. After centrifugation at 4400 g for 10 min at 4 °C, the insoluble fraction was separated from the supernatant. The pellet including integral membrane proteins was resuspended in TD buffer supplemented with 2 % SDS. The mixture was shaken at 15 rev./min for 1 h at room temperature (25 °C) to solubilize the membrane proteins and centrifuged at 13 000 g for 15 min. The supernatant was analysed by Western blotting using antibodies against GFP (Roche Diagnostics).

Protein extracts were diluted in loading buffer (60 mM Tris/HCl, pH 6.8, 2 % SDS, 100 mM DTT, 10 % glycerol and 0.1 % Bromophenol Blue) and boiled for 5 min. Recombinant GFP (Roche Diagnostics) was used as a control and was subjected to the same treatment as the rest of the samples. Proteins were run in a 12 % acrylamide gel, which were electroblotted on to nitrocellulose filters by applying a current of 0.65 mA/cm² of gel for 2 h. A protein mixture ‘Precision Plus Protein All Blue Standards’ (Bio-Rad Laboratories) was used as molecular-mass markers. Membranes were blocked by incubation in 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.2 % Tween 20 and 5 % (w/v) non-fat dried milk powder for 2 h at room temperature. Primary mouse monoclonal anti-GFP antibodies were added to this buffer (1/1000 dilution), and the blot was incubated at 4 °C overnight. Filters were washed thoroughly in 50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.2 % Tween 20, and incubated with the anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich). The reaction signal was detected using NBT (Nitro Blue Tetrazolium) and BCIP (5-bromo-4-chloroindol-3-yl phosphate) (Roche Diagnostic) as substrates.

CPC synthesis in cell-free extracts

Mycelia from *A. chrysogenum* cultures in DP medium were collected at 144 h, washed with 0.9 % NaCl and disrupted by grinding them in a mortar with liquid nitrogen. Cell extracts were resuspended in PP buffer (50 mM potassium phosphate, pH 7.0) and centrifuged at 16 000 g for 10 min at 4 °C. Cell-free extracts (500 μl) were incubated at 25 °C on a shaker at 250 rev./min to provide O₂ for the oxygen-dependent expandase/hydroxylase reactions. Samples of 100 μl were collected after 1 h, and the reaction was stopped by treatment with 1 vol. of methanol. The in vitro formation of CPC was determined by HPLC. In the absence of added PenN, this reaction measures the conversion of endogenous PenN (released by disruption of microbodies) into CPC.

Fluorescence microscopy

The fluorescence emissions of hyphae and arthrospores were analysed by confocal laser-scanning microscopy using a Radiance 2000 Laser Confocal Microscope (Bio-Rad Laboratories). GFP fluorescence was visualized with a number 13 filter (470 nm excitation, 20 nm bandwidth; 505–530 nm emission).

RESULTS

Identification of ORF10 downstream of the *cefD1* gene

Transmembrane proteins, other than CefT, that might be β-lactam exporters were searched for in the DNA regions adjacent to the cephalosporin gene clusters. To extend the cloned *cef* region of chromosome VII, a gene library of the *A. chrysogenum* C10 strain [4] was screened with a probe internal to the ‘early’ gene cluster (330 bp SalI/EcoRV fragment of the *cefD1* gene). Six phage plaques gave positive hybridization with this probe. They corresponded to phages F2, F3, F4, F5, F6 and F8 with six different inserts of *A. chrysogenum* DNA. Sequence analysis of the region downstream of the *cefD1* gene (of phage F8) revealed the presence of a new ORF named ORF10.

An EcoRV fragment (3.1 kb long) of the region located downstream of the *cefD1* gene was cloned into pZErO2 in both orientations, giving rise to plasmids pcefMa and pcefMb. The inserts pcefMa and pcefMb were sequenced completely on both strands. The nucleotide sequence of this fragment was deposited in the nucleotide sequence databases under the accession number AM231815 (cefM). Analysis of the nucleotide sequence of the 3.1 kb DNA insert revealed the presence of one complete ORF, initially named ORF10. This ORF was 1620 nt long and was interrupted by the presence of three introns. The presence of the introns was confirmed by RT–PCR.

ORF10 encodes a protein with high similarity to multidrug efflux pump proteins

ORF10 encodes a protein of 482 amino acids with a deduced molecular mass of 52.2 kDa. The amino acid sequence of the ORF10 protein showed strong similarity throughout its entire length to multidrug efflux pump membrane proteins belonging to the MFS. The amino acid sequence of the ORF10 protein showed motifs A, B, C, D2 and G characteristic of the drug/H⁺ antiporter 12 TMS (transmembrane segment) group of the MFS [30]. This protein is similar to MFS proteins (Figure 1) of *Neosartorya fischeri* (48.9 % identical amino acids), *Aspergillus fumigatus* (47.8 % identical amino acids) and *Aspergillus clavatus* (46.5 % identical amino acids). Also, the ORF10 protein shared 31.9 %
Figure 1  Alignment of the deduced amino acid sequences of protein encoded by the cefM gene of A. chrysogenum with the protein encoded by the cefT gene of A. chrysogenum and the hypothetical MFS proteins of A. clavatus (GenBank® accession number XP_001273827), A. fumigatus (GenBank® accession number XP_748433) and N. fischeri (GenBank® accession number XP_001258746)

The 12 TMSs are overlined with a solid bar, and the characteristic motifs (A, B, C, D 2 and G) of the MFS are overlined with thin lines. Only the identical amino acids are shaded. The proteins were aligned using the ClustalX program. Note that the amino acid sequence of the Pex19-binding site in CefM protein (residues 212–221) is boxed.

The 12 TMSs are overlined with a solid bar, and the characteristic motifs (A, B, C, D 2 and G) of the MFS are overlined with thin lines. Only the identical amino acids are shaded. The proteins were aligned using the ClustalX program. Note that the amino acid sequence of the Pex19-binding site in CefM protein (residues 212–221) is boxed.

The 12 TMSs are overlined with a solid bar, and the characteristic motifs (A, B, C, D 2 and G) of the MFS are overlined with thin lines. Only the identical amino acids are shaded. The proteins were aligned using the ClustalX program. Note that the amino acid sequence of the Pex19-binding site in CefM protein (residues 212–221) is boxed.

identical amino acid residues with the previously reported CefT protein (CAD32176) of A. chrysogenum [15]. The low percentage of identity between cefT and the ORF10 protein suggests that they transport different substrates in this fungus.

Computer analysis of the predicted ORF10 protein with the TopPred2-Topology program of the membrane protein database (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=toppred) indicated that it contains 12 TMSs. In the predicted membrane topology, the C- and N-terminal ends are in the internal face of the membrane, a characteristic of MFS proteins [30,31]. Therefore the gene encoded by ORF10 has been named cefM (for microbody membrane protein).

Pex19 is a peroxin that interacts with peroxisomal membrane proteins for their import. Analysis of Pex19-binding sites (http://www.peroxisomedb.org/) in the CefM protein showed one putative Pex19-binding site [26] between the amino acids
CefM transporter is essential for cephalosporin biosynthesis

Figure 2 Disruption of cefM by a double crossing-over recombination and molecular analysis of the transformants

(A) Scheme of disruption of cefM by gene replacement with the pDM plasmid. The size of fragments expected after the double crossing-over is indicated. (B and C) Southern blot hybridizations of EcoRI-digested genomic DNA from several transformants with labelled probes internal to the cefM (B) or ble (C) genes. Lane M, size markers (lambda DNA/HindIII digested); lane 1, TDM-38; lane 2, TDM-57; lane 3, TDM-64; lane 4, TDM-87; lane 5, TDM-131; lane 6, TDM-139; lane 7, TDM153; lane 8, A. chrysogenum C10. Note that transformants TDM-87 and TDM-139 contain an intact pDM construction (lanes 4 and 6). Transformant TDM-139 shows the correct gene replacement (lane 6). The sizes of the hybridization bands (in kb) are indicated on the right.

212 and 221 (Figure 1). This observation suggested that CefM may be a peroxisome (microbody) membrane protein [32] (see below).

Targeted inactivation of cefM results in a drastic reduction in cephalosporin production

To determine whether the CefM protein is involved in CPC biosynthesis or transport, we inactivated this ORF by double recombination with plasmid pDM (Figure 2A). Transformants of A. chrysogenum C10 with this plasmid were selected by resistance to phleomycin and screened using the double-marker technique [7,15,17]. Clones showing a hyg<sup>+</sup> (hygromycin-sensitive) phle<sup>-</sup> (phleomycin-resistant) phenotype, indicating that double recombination had occurred, were selected. To confirm that targeted inactivation had occurred at the correct position, seven transformants and the parental strain A. chrysogenum C10 (positive control), were analysed by Southern blot hybridization (Figure 2B and 2C) with a 608 bp SalI fragment of the cefM gene as probe (Figure 2A). Results showed that the parental strain of A. chrysogenum C10 (Figure 2B, lane 8) hybridized with a genomic DNA band of 3.8 kb; however, in the TDM-139 (for
transformant disrupted in *cefM* strain, the 3.8 kb hybridization band was converted into one band of 5.3 kb, as expected, by a canonical double recombination (Figure 2B; lane 6). A 5.3 kb hybridization signal was found when the *ble* gene was used as probe as expected, confirming that the endogenous *cefM* gene had been disrupted (Figure 2C, lane 6). Other transformants still showed the original 3.8 kb band (see Figures 2B, lane 4), indicating that *cefM* gene replacement had not occurred and the plasmid integration was ectopic.

The TDM-139 strain and parental *A. chrysogenum* C10 as control strain were cultured in defined cephalosporin production DP medium. Results of the fermentations showed that the TDM-139 strain growth rate is similar to that of the parental strain until 72 h, and thereafter the TDM-139 strain did not show the characteristic lysis phase of the parental strain (Figure 3A). Analyses of the culture broth supernatant by bioassay (Figure 3B) and HPLC (Figure 3C) showed that the specific CPC biosynthesis in the disrupted strain was drastically reduced. Also, analysis by
HPLC of TDM-139 culture broth supernatant showed a 91.5% reduction in the specific production of extracellular penicillins (IPN and PenN) (Figure 3D). Further HPLC analysis of the residual penicillins (see below) showed that the penicillin in this mixture was mostly PenN (see below).

**The TDM-139 strain accumulates intracellular PenN**

To study whether the protein encoded by the cefM gene was involved in PenN transport to microbodies or to the culture medium, the intracellular PenN levels were measured in cell extracts of the TDM-139 strain and the parental strain A. chrysogenum C10 grown in DP medium for 96, 120 and 144 h. Cell-free extracts of both A. chrysogenum strains were treated with GITC (2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate) and the derivatized penicillin compounds resolved by HPLC as described previously [7,33]. Results showed relatively high intracellular PenN levels in the TDM-139 strain at 96, 120 and 144 h compared with the parental strain (Figure 3E). The intracellular PenN levels were approx. 7-fold higher in the TDM-139 strain than in the parental strain A. chrysogenum C10. Taken together, these results indicate that the cefM mutant is unable to synthesize cephalosporin owing to a lack of PenN transport related to its conversion into cephalosporin and, as a result, a significant amount of intracellular PenN is accumulated.

**Complementation of the cefM mutation restores CPC biosynthesis and normal intracellular PenN levels**

For complementation studies, pCM bearing the intact cefM gene with its own promoter (Figure 4A) was transformed into the TDM-139 mutant, and transformants were selected by resistance to hygromycin. Five transformants that showed resistance to hygromycin were selected at random, and their EcoRI-digested genomic DNA was hybridized with a 608 bp fragment of the cefM gene as probe (Figure 4A). Results showed that all five transformants (Figure 4B, lanes 1–5) hybridized with a DNA band of 4.8 kb in addition to the endogenous 5.3 kb hybridization band (corresponding to the disrupted cefM gene in the host TDM-139 mutant; Figure 4B, lane 6), indicating that the insertion of plasmid pCM had occurred without reorganization in the transformants.

The effect of complementation of cefM on CPC production was studied with three different TCM (for transformant complemented in cefM) strains. Results showed that in transformants TCM-27, TCM-28 and TCM-35, the growth rate (Figure 3A) and the specific cephalosporin production (Figures 3B and 3C) were restored to levels similar to those of the parental A. chrysogenum C10 strain. Analysis of extracellular (Figure 3D) and intracellular (Figure 3E) penicillin levels by HPLC showed that the PenN level was restored in the three TCM transformants to the same low level as in the parental strain A. chrysogenum C10 (Figure 3E).

Since complementation with cefM restores cephalosporin production, it is clear that all other genes of the cephalosporin pathway are functional.

**Disruption of cells releases compartmentalized PenN allowing in vitro conversion into cephalosporins**

All other genes of the cephalosporin pathway appeared to be functional in the cefM-disrupted mutant. This was confirmed by the in vitro conversion of PenN into CPC using cell-free extracts of the transformants TDM-139, TCM-27 and the parental strain grown in DP medium for 144 h. Results showed that the expandase/hydroxylase (converting PenN into DAC) and DAC acetyltransferase (converting DAC into CPC) activities were present in the disrupted strain as well as in the others, because

**Table 1 CPC synthesis in cell-free extracts of the transformants TDM-139 and TCM-27 (derived from TDM-139 by complementation with the cefM gene)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>CPC production (ng/mg of mycelia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. chrysogenum C10</td>
<td>Wild-type</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>A. chrysogenum TDM-139</td>
<td>cefM−</td>
<td>17.8 ± 2.0</td>
</tr>
<tr>
<td>A. chrysogenum TCM-27</td>
<td>Wild-type</td>
<td>7.1 ± 0.5</td>
</tr>
</tbody>
</table>
CefM. For this propose, the TDM-139 mutant (disrupted in the cefM gene) was transformed with plasmid pcefM-gfp (Figure 5A). This plasmid contains the cefM promoter (1 kb) upstream of a cefM-gfp hybrid gene that encoded a protein consisting of the 482 amino acids of the CefM fused in-frame to the N-terminal end of GFP.

The pcefM-gfp transformants were tested for cephalosporin production by the agar plug method obtaining two transformants that showed a similar cephalosporin production to that of A. chrysogenum C10. Total DNA was extracted from the blocked mutant, from these two transformants, from mutant TDM-139 and from the parental strain A. chrysogenum C10; the DNAs were digested with a mixture of BamHI and MluI and hybridized with a 608 bp SalI probe of the cefM gene. Results showed (Figure 5B) that the probe hybridized with a band of 3.3 kb, as expected (Figure 5B, lanes 1 and 2), indicating that the cefM-gfp hybrid gene was integrated in a non-reorganized form. A 7.4 kb hybridization band was observed in the cefM disrupted mutant used as host strain (Figure 5B, lane 3), whereas the 8.3 kb hybridization band corresponds to the wild-type strain (Figure 5B, lane 4). The fused CefM–GFP in these two transformants is functional since it restored cephalosporin production to the blocked mutant.

The fused CefM–GFP remains intact in the integral membrane protein fraction of a TcefM–GFP transformant

To exclude the possibility that the GFP might be cleaved from the fused CefM–GFP, Western blot analysis (Figure 5C) of the pellet including integral membrane proteins was carried out using extracts of the TcefM–GFP36 transformant (Figure 5C, lane 3) and the untransformed mutant TDM-139 (Figure 5C, lane 2) and the parental strain A. chrysogenum C10 (Figure 5C, lane 1). The results showed unequivocally that the CefM–GFP fusion protein remained intact. The fused CefM–GFP showed an estimated molecular mass of 79.2 kDa (Figure 5C, lane 3), as expected, whereas a control sample containing free GFP (Figure 5C, lane 4) showed a molecular mass of 27 kDa. No GFP was observed in untransformed strains (Figure 5C, lanes 1 and 2).

The CefM–GFP hybrid protein is located in intracellular microbodies

The TcefM–GFP36 transformant was used to determine the subcellular localization of the CefM–GFP hybrid protein (Figure 6). The parental and the complemented strains grow and differentiate into typical arthrospore chains that are associated with high cephalosporin production (see the Discussion) (Figure 6A). However the formation of arthrospores in the disrupted strain TDM-139 was impaired (Figure 6B). Comparison of phase-contrast (Figure 6C, panel I), fluorescence images (Figure 6C, panel II) and merged images (Figure 6C, panel III) showed that the CefM–GFP hybrid protein was targeted to a type of medium-sized microbodies, where they produce punctate fluorescence, as shown in the enlarged photograph of one hypha (Figure 7). This kind of fluorescence was characteristic of the GFP–PTS fusions that targeted to peroxisomes [34]. The abundance of these microbodies in the A. chrysogenum C10 strain is probably related to the high level of cephalosporin production in this improved mutant strain.

DISCUSSION

Compartmentalization of specific reactions of the β-lactam biosynthesis pathways provides a way to control this pathway by
CefM transporter is essential for cephalosporin biosynthesis

Figure 6  Subcellular localization of the CefM protein

Hyphae and arthrospore chains of A. chrysogenum C10 (A), the disrupted mutant TDM-139 (B) and the complemented TcEFM–GFP36 (C). Strains were observed by phase-contrast microscopy (row I) or fluorescence microscopy (row II), and images were merged (row III). Samples were obtained from cultures grown for 72 h in CCM medium. The fluorescence was monitored for 96 h. Note that the cefM-disrupted mutant (B) shows impaired differentiation into arthrospores.

Figure 7  Enlarged detail of a hyphal fragment showing the localization of the CefM–GFP-labelled microbodies

Culture condition and staining procedures were as in Figure 6.
holding together enzymes with their substrates inside organelles [12]. In *Penicillium chrysogenum*, specific steps of the β-lactam biosynthesis pathway are compartmentalized; the first two enzymes (ACV synthetase and IPN synthase) occur in the cytosol [12,13,35] and the last step of the pathway in peroxisomes [13,14,36].

In *A. chrysogenum*, several enzymes of the cephalosporin biosynthesis pathway, including ACV synthetase [37], IPN synthase [38], DAOc synthase-hydroxylase [39,40] and DAC acetyltransferase [5,41] seem to be located in the cytosol [11,12]. A central step of this biosynthetic pathway is the poorly known conversion of IPN into PenN [33] that is catalysed by the isopenicillinyl N-CoA synthetase and isopenicillinyl-CoA epimerase proteins encoded by the *cefD1* and *cefD2* genes respectively [7]. The PTSs allow the sorting of peroxisomal matrix proteins. There are two types of PTS: the C-terminal PTS-1 with consensus sequence SKL (Ser-Lys-Leu) and the N-terminal nonapeptide PTS-2 with consensus sequence (R/L)(L/V/I)-X5-(H/Q)(L/A) [19]. We have observed that CefD1 and CefD2 proteins contain a C-terminal PTS-1 for peroxisomal localization [42]. CefD1 contains a putative PTS-1, whereas CefD2 contains both PST1 and PST2 signals. Jayatilake et al. [43], Baldwin et al. [44] and Lübbe et al. [45] studied the *in vitro* conversion of isopenicillin to PenN in cell-free extracts and found that it was optimal at pH 7; this same pH was reported for the peroxisomal lumen of *P. chrysogenum* that is slightly higher than that of the cytosol [46]. Therefore the epimerization of IPN to PenN seems to take place in peroxisomes and requires specific transport steps of intermediates through peroxisomal membranes. The presence of *cefT* [15] and *cefM* in the cephalosporin gene clusters is consistent with an increasing body of evidence showing that export genes are frequently linked to antibiotic biosynthesis genes [47].

The secretion of different secondary metabolites from the producer strains is a subject of interest [48]. Despite the industrial relevance of many of the secondary metabolites, only in a few cases have the membrane proteins involved in secretion of those metabolites been identified and validated by gene disruption. Particular attention should be given to the transporters involved in penicillin or cephalosporin secretion, because of the very large amounts of these β-lactams that need to be secreted in the industrial strains.

It is clear from the results of this work that the *cefM* gene located downstream of the *cefD1* gene encoding the proposed isopenicillinyl N-CoA ligase of the two-component epimerization system [7,33] is essential for cephalosporin biosynthesis. The CefM protein belongs to the MFS, particularly to Family 3 (drug efflux proteins), the same family as the CefT protein [15]. MFS transporters are single-polypeptide secondary carriers (drug efflux proteins), the same family as the CefT protein CefM protein belongs to the MFS, particularly to Family 3 [15].

TheAuthors Journal compilation © 2009 Biochemical Society

Figure 8 Model of localization of the different steps of the CPC biosynthetic pathway in *A. chrysogenum* showing the localization of the CefM transporter (shaded) in the membrane of microbodies (MB)

The microbody location of CefM was fully supported by the results of the fluorescent microscopy analysis using a functional CefM–GFP fusion. Using high-resolution phase-contrast and fluorescence microscopy, the fusion protein was found to be located in medium-sized microbodies in the hyphae and particularly in the arthrospore chains of *A. chrysogenum*. The fluorescent microbodies were abundant in differentiating *A. chrysogenum* cells after 72 h of cultivation, coinciding with the phase of active cephalosporin biosynthesis.

This finding is very interesting since, for many years, it has been known that there is a direct correlation between arthrospore formation and the CPC biosynthetic ability [49]. It is now clear from the results of the present study that the CefM-containing microbodies are particularly abundant in hyphae that are starting to differentiate and in the arthrospores themselves.

The microbodies that we observed are very similar to the peroxisomes identified in *P. chrysogenum* that contain a PST-1 peroxisomal-targeted GFP [34]. However, we do not exclude the possibility at this time that the fluorescent microbodies might be a more specific cephalosporin biosynthesis-related type of ‘lipid bodies’.

The substrate transported by the CefM protein out of the microbodies appears to be PenN. Since ACV and IPN are synthesized in the cytosol by cytoplasmic ACV synthetase and IPN synthase [12,50], the IPN has to be transported into peroxisomes or microbodies, as it occurs in *P. chrysogenum*, during penicillin biosynthesis [51]. After epimerization by the two-component CefD1–CefD2 peroxisomal epimerization system in *Acremonium*, PenN is likely to be transported back into the cytosol, since the three last enzymes of the cephalosporin pathway have been reported to be soluble [11,12].

The entry of IPN into peroxisomes seems to be coupled to its activation to isopenicillinyl N-CoA by the CefD1 acyl-CoA ligase. Very-long-chain acyl-CoA ligases similar to CefD1 are known to be involved in transport of fatty acids. Therefore the exit of an early intermediate of the pathway to the cytosol is the most likely transport step performed by CefM (Figure 8). This role is consistent with the increase in intracellular PenN and the drastic reduction of the extracellular PenN and of formation of cephalosporin in the TDM-139 mutant disrupted in *cefM*. If the PenN can not be transported out of the microbody, the entire pathway is blocked, and the early steps of the pathway are feedbackinhibited.

The *cefT* and *cefM* genes are located in the opposite ends of the ‘early’ cephalosporin gene cluster, and encode transporters with clearly different functions. Mutants disrupted in *cefT* still produce normal levels of cephalosporin, whereas, as shown in the present study, a mutant disrupted in *cefM* fails to produce cephalosporin, although it contains the enzymes required for the conversion of PenN into CPC. The CefT protein was proposed to be involved in cephalosporin secretion from the cell [15], whereas CefM is clearly located in microbodies and is involved in the transport of an early intermediate of the cephalosporin pathway.

In summary, the cephalosporin pathway in *A. chrysogenum* provides a good example of compartmentalization of precursors and enzymes. Therefore the CefM transporter plays a critical
role in their proper localization and is essential for cephalosporin biosynthesis.

ACKNOWLEDGEMENTS

We acknowledge the support of Dr Roel Bovenberg, DSM (Delft, The Netherlands) and the excellent technical assistance of A. Sánchez, B. Martín, J. Merino, A. Caseneuve and B. Aguado.

FUNDING

This work was supported by grants of the European Union [grant number Eurofung 015387] and DSM (Delft, The Netherlands). F. T. received a FPU (Formación de Profesores Universitarios) predoctoral fellowship from the Ministry of Education and Science (Madrid). C. G.-E. is supported by the Torres Quevedo Program [grant number PTQ04-3-0411].

REFERENCES


Received 11 June 2008/3 October 2008; accepted 8 October 2008
Published as BJ Immediate Publication 8 October 2008, doi:10.1042/BJ20081180