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

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45 **SHORT TITLE:** CefM transporter is essential for cephalosporin biosynthesis

SYNOPSIS

50 The cluster of early cephalosporin biosynthesis genes (*pcbAB*, *pcbC*, *cefD1*, *cefD2* and
cefT of *Acremonium chrysogenum*) contains all 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 family with 12 transmembrane spanning domains, different from the
55 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-,
deacetylcephalosporin C and cephalosporin C and shows impaired differentiation into
arthrospores. Complementation of the disrupted mutant with the *cefM* gene restored the
60 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 protein 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
65 intense cephalosporin biosynthesis. Since the dual component enzyme system CefD1-
CefD2 that converts isopenicillin N to penicillin N contains peroxisomal targeting
sequences, it is probably 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
70 to cephalosporin C.

75 **Keywords:** *Acremonium chrysogenum*, cephalosporin C biosynthesis, CefM transporter,
translocation of penicillin N from microbodies, compartmentalization.

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Abbreviations used:

CCM, complex culture medium; CPC, cephalosporin C; DAC, deacetylcephalosporin
C; DP, Shen's defined production medium; DTT, dithiothreitol; GFP, Green fluorescent
85 protein; HPLC, high performance liquid chromatography; IPN, isopenicillin N; ORF,
open reading frame; PenN, penicillin N; Pex19, peroxisome biogenesis factor 19; PTS1,
peroxisomal targeting signal 1; PTS2, peroxisomal targeting signal 2; RT-PCR, reverse
transcription-polymerase chain reaction.

INTRODUCTION

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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 *Kallicrhoma tethys*, a marine fungus in which some genes for cephalosporin biosynthesis have been reported recently.

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The genes involved in 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 synthetase, *pcbC* (isopenicillin N synthase), *cefD1* (isopenicillinyl-CoA synthetase), *cefD2* (isopenicillinyl-CoA epimerase)] located in chromosome VII (4.6 Mb) and ii) a 'late' cephalosporin cluster [*cefEF* encoding deacetoxycephalosporin C synthase (expandase) /deacetylcephalosporin C hydroxylase, *cefG* (acetyl-coenzyme A: deacetylcephalosporin C acetyltransferase)] in chromosome I (2.2 Mb) [4-7].

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This arrangement of the cephalosporin genes in two separate clusters is intriguing and contrast with the organization in *Penicillium chrysogenum* where all 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 evolutionarily assembled from two different parts encoded by separate DNA fragments [10].

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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 isopenicillin N) occur in the cytosol [11,12], whereas the last steps (activation of phenylacetic acid to phenylacetyl-CoA and the conversion of isopenicillin N to benzylpenicillin) occur in peroxisomes [13,14].

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In *A. chrysogenum*, it was initially believed that all steps have a cytosolic location [11,12]. However, recent evidence suggests that the epimerization of isopenicillin N (IPN) to penicillin N (PenN) is catalyzed 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.

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In *A. chrysogenum* the previously reported *cefT* gene [15] is located in the early cephalosporin cluster (chromosome VII) downstream from *pcbAB* and encodes a multidrug efflux pump protein belonging to the Major Facilitator Superfamily (MFS) 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 two-fold increase in cephalosporin C 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.

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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 this article, we report the characterization of a new gene *cefM* that encodes a membrane protein belonging to the MFS family and its essential role in β -lactam transport.

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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 by 19]. There are
140 several types of peroxisomal targeting sequences: for matrix proteins (PTS-1 and PTS-
2) [19] and for membrane proteins [20,21]. These targeting sequences of peroxisomal
membrane proteins, contain a basic amino acid combined with at least one
transmembrane region [22-24]. These proteins are recruited from the cytoplasm by the
145 Pex19p [25] and the consensus sequences for binding to Pex19p have been identified
[26].

It was therefore of interest to study if the MDR transmembrane proteins encoded by
genes located in the 'early' cephalosporin cluster are targeted to the peroxisomal
membrane. We described in this article for the first time, the compartmentalization in
microbodies of one part of the cephalosporin C biosynthesis pathway.

150

EXPERIMENTAL

155 **Microorganism and culture media**

Acremonium chrysogenum C10 (ATCC 48272) a high-cephalosporin-producing strain
available from PanLabs was used in this study. For sporulation *A. chrysogenum* C10
and the mutant strains were grown in solid LPE medium [7,15] for 7 days at 28 °C.
160 Spores and mycelium fragments collected from six plates of LPE medium were
suspended in 100 ml of seed medium [7] in 500 ml shake-flasks and incubated at 25 °C
for 48 h in an orbital incubator at 250 rpm. Ten ml of seed culture was used to inoculate
100 ml of Shen's defined production (DP) medium. The cultures were incubated in
triple-baffled flasks (500 ml; Bellco) containing 100 ml of medium at 25°C in a rotary
165 shaker (250 rpm). Samples were taken every 24 h, and cephalosporin production was
determined by bioassays against *Escherichia coli* ESS2231 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 medium.

170

Library screening

A gene library of *A. chrysogenum* C10 was constructed in the ble-EMBL3 vector as
described before (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
175 infection plates was transferred onto Hybond-NX membranes (Amersham-Biosciences).
In order to find the positive phages with the new open reading frame, two or three
consecutive rounds of hybridizations were done using the appropriate digoxigenin labelled
probe according the Dig Easy Hyb system (Roche Diagnostic Corporation) as described
by Ullán et al. [15]. Finally, the DNA fragment containing the *cefM* gene was cloned
180 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
185 with restriction enzymes and separated in 0.7% agarose gels. The gels were blotted onto
Hybond-NX membranes (Amersham-Biosciences) as described by Ullán et al. [15]. For
Southern blot analysis the Dig Easy Hyb system (Roche Diagnostic Corporation) was
used. Hybridizations were performed according to the manufacturer's protocol and the

190 hybridization signals were visualized with chemiluminescence and recorded on X-ray film with an exposure time of 5 min.

Plasmids containing the *cefM* gene

195 *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 (a or b) at the unique *EcoRV* site of pZErO-2 (Invitrogen)

200 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.

205 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.

210 *pcefM-GFP*. To obtain the fused *cefM-gfp* gene an *Ecl136II* 2.6 kb DNA-fragment obtained from plasmid *pcefMa* by PCR using the M13 Forward and MC (5'-GAGCTCCCACAGCGCTGGCTGAA-3') oligonucleotides, was inserted into the *Ecl136II* site of pMCB15 bearing the *gfp2.5* gene of *Aquarea victoria* [28].

DNA sequencing

215 Sequencing reactions of the DNA were made by standard procedures [15] and automatic sequencing was performed with the AutoReadTM System (Pharmacia, Uppsala, Sweden).

RNA extraction and intron analysis

220 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 introns splicing sites was amplified by RT-PCR (Promega) using RNA of *A. chrysogenum* 48 h cultures as template with the primer pairs I1-R: 5'-GCTTTGGAGGCGGCGATAGACA-3' and I1-F: 5'-GGCGGGGAGGTTGGGACTGA-3'; I2-R: 5'-GTGCGCCCTCAGTCCCAACCT-3' and I2-F: 5'-TGAGAAGACGACGGCGATGAAGAC-3'. I3-R: 5'-CGCCGTCGTCTTCTCATACCAGTT-3' and I3-F: 5'-CCACGGCAATGAATCCAAGAGTCG-3'. The amplified regions were sequenced to confirm the presence of the 3 introns.

A. chrysogenum transformation

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

235 HPLC determination of isopenicillin N, penicillin N, deacetylcephalosporin C and cephalosporin C

The extracellular and intracellular concentrations of isopenicillin N (IPN), penicillin N (PenN), deacetylcephalosporin C (DAC) and cephalosporin C (CPC) of *A. chrysogenum*

240 strains were determined in a Waters HPLC System equipped with a 2487 dual absorbance detector, using a 4.6 x 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

245 Mycelia samples from the different *A. chrysogenum* strains were centrifuged for 10 min at 4,400 x g 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 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 4,400 x g for 10 min at 4°C, the insoluble fraction was separated from the 250 supernatant. The pellet including integral membrane proteins was resuspended in TD buffer supplemented with 2% SDS. The mixture was shaken at 15 rpm for 1 h at room temperature to solubilize the membrane proteins and centrifuged at 13,000 x g for 15 min. The supernatant was analysed by western blotting, using antibodies against GFP (Roche Diagnostic).

255 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. The recombinant GFP protein (Roche Diagnostics Corp., Indianapolis, IN, U.S.A.) was used as control and was subjected to the same treatment than the rest of the samples. Proteins were run in a 12% acrylamide gel, which were electroblotted onto nitrocellulose filters applying a current of 0.65 mA/cm² of gel for 2 h. A protein mixture “Precision Plus Protein All Blue Standards” (Bio-Rad, Hercules, CA, USA) was used as molecular weight markers. Membranes were blocked by incubation in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% Tween 20 and 5% nonfat milk powder (w/v) for 2 h at room temperature. Primary mouse monoclonal anti-GFP antibodies were added to this buffer 265 (1/1000 dilution), and the blot was incubated overnight. Filters were washed thoroughly in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20 and incubated with the anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich). The reaction signal was detected using nitro-blue tetrazolium chloride and 5-bromo-4 chloro-3 indolyl-phosphate (Roche Diagnostic) as substrates.

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Cephalosporin C synthesis in cell-free extracts

275 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 TD buffer (50 mM KH₂PO₄ pH 7.0) and centrifuged at 14000 rpm for 10 min at 4°C. Cell-free extracts (500 µl) were incubated at 25°C on a shaker at 250 rpm to provide O₂ for the oxygen-dependent expandase/hydroxylase reactions. Samples (100 µl) were collected at 1 h, and the reaction was stopped by treatment with 1 volume of methanol. The *in vitro* formation of cephalosporin C was determined by HPLC. In absence of added PenN this reaction measures the conversion 280 of endogenous PenN (released by disruption of microbodies) into cephalosporin C.

Fluorescence microscopy

285 The fluorescence emissions of hyphae and arthrospores were analyzed by confocal laser scanning microscopy using a Laser Confocal Microscope RADIANCE 2000 (BIO-RAD). Green fluorescent protein (GFP) fluorescence was visualized with a number 13 filter (470/20 nm; 505-530 nm).

RESULTS

Identification of the ORF 10 downstream of the *cefD1* gene

290 Transmembrane proteins, other than CefT, that might be β -lactam exporters were searched 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 open reading frame named ORF10. An *EcoRV* fragment (3.1 kb long) of the region located downstream of the *cefD1* gene was cloned into the pZero2 in both orientations, giving rise to plasmids *pcefMa* and *pcefMb*. The inserts *pcefMa* and *pcefMb* were completely sequenced on both strands. The nucleotide sequence of this fragment was deposited in the EMBL database 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 as indicated in Experimental Procedures.

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

310 The ORF10 encoded a protein of 482 amino acids with a deduced molecular mass of 52.2 kDa. The amino acid sequence of the ORF10p showed strong similarity throughout its entire length to multidrug efflux pump membrane proteins belonging to the Major Facilitator Superfamily (MFS). The amino acid sequence of the ORF10p showed motifs A, B, C, D2 and G characteristic of the Drug: H⁺ antiporter 12-TMS group of the major facilitator superfamily [30]. This protein is similar to MFS proteins (Fig. 1) of *Neosartorya fischeri* (48.9 % identical amino acids), *Aspegillus fumigatus* (47.8 % identical amino acids) and *Aspergillus clavatus* (46.5 % identical amino acids). Also, the ORF10 protein shared 31.9 % identical amino acid residues with the previously reported CefT protein (CAD32176) of *A. chrysogenum* [15]. The low percentage of identity between *cefT* and ORF10p suggests that they transport different substrates in this fungus.

320 Computer analysis of the predicted ORF10 protein with the TopPred2-Topology program of the membrane protein database (<http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred.html>) indicated that it contains 12 transmembrane spanning (TSM) domains. In the predicted membrane topology, the carboxyl and amino ends are in the internal face of the membrane, that it is a characteristic of MFS proteins [30,31]. Therefore, the gene encoded by ORF10 has been named *cefM* (for microbody membrane protein).

330 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 212 and 221 (Fig. 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

335 To determine if the CefM protein is involved in cephalosporin C biosynthesis or transport, we inactivated this ORF by double recombination with plasmid pDM (see Experimental Procedures) (Fig. 2A). Transformants of *A. chrysogenum* C10 with this

340 plasmid were selected by resistance to phleomycin and screened by the double marker
technique [7,15,17]. Clones showing a hyg^S $phle^R$ phenotype, indicating that double
recombination had occurred, were selected. To confirm that targeted inactivation had
occurred at the right position, seven transformants and the parental strain *A.*
chrysogenum C10 (positive control), were analyzed by Southern blot hybridization (Fig.
2B and C) with a 608 bp *SalI* fragment of the *cefM* gene as probe (Fig. 2A). Results
345 showed that the parental strain *A. chrysogenum* C10 (Fig. 2B, lane 8) hybridized with a
genomic DNA band of 3.8 kb; however, in the transformant TDM-139 (for
transformants disrupted in *cefM*), the 3.8 kb hybridization band was converted into one
band of 5.3 kb, as expected, by a canonical double recombination (Fig. 2B; lane 6). A
5.3 kb hybridization signal was found when the *ble* gene was used as probe as expected,
350 confirming that the endogenous *cefM* gene has been disrupted (Fig. 2C, lane 6). Other
transformants still showed the original 3.8 kb band (see Fig. 2B, lane 4), indicating that
cefM gene replacement had not occurred and the plasmid integration was ectopic.
The disrupted mutant TDM-139 and *A. chrysogenum* C10 as control strain were
cultured in defined cephalosporin production DP medium. Results of the fermentations
355 showed (Fig. 3A) that the TDM-139 strain growth rate is similar to that of the parental
strain until the 72 hours and thereafter the TDM-139 strain did not show the
characteristic lysis phase of the parental strain. Analyses of the culture broth supernatant
by bioassay (Fig. 3B) and HPLC (Fig. 3C) showed that the specific cephalosporin C
biosynthesis in the disrupted strain was drastically reduced. Also, analysis by HPLC of
360 TDM-139 culture broth supernatant showed a 91.5 % reduction in the specific
production of extracellular penicillins (IPN and PenN) (Fig. 3D). Further HPLC
analysis of the residual penicillins (see below) showed that the penicillin in this mixture
was mostly penicillin N (see below).

365 **The TDM-139 strain accumulate intracellular PenN**

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

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

For complementation studies plasmid pCM bearing the intact *cefM* gene with its own
promoter (Fig. 4A) was transformed into the TDM-139 mutant and transformants were
selected by resistance to hygromycin. Five transformants that showed resistance to
385 hygromycin were selected at random, and their *EcoRI*-digested genomic DNA was
hybridized with a 608 pb fragment of the *cefM* gene as probe (Fig. 4A). Results showed
that all five transformants (Fig. 4B; lanes 1, 2, 3, 4 and 5) gave hybridization 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; Fig. 4B, lane 6), indicating that the insertion of plasmid pCM had occurred without reorganization in the transformants.

The effect of complementation of *cefM* on cephalosporin C production was studied with three different TCM transformants (for transformants complemented in *cefM*). Results showed that in transformants TCM-27, TCM-28 and TCM-35, the growth rate (Fig. 3A) and the specific cephalosporin production (Fig. 3B and C) was restored to levels similar to those of the parental *A. chrysogenum* C10 strain. Analysis of extracellular (Fig. 3D) and intracellular (Fig. 3E) penicillin levels by HPLC showed that the penicillin N level was restored in the three TCM transformants to the same low level as in the parental strain *A. chrysogenum* C10 (Fig. 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 to 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 to CPC using cell-free extracts of the transformants TDM-139 (disrupted in *cefM*), TCM-27 (complemented) and the parental strain grown in DP medium for 144 h. Results showed (Table 1) that the expandase/hydroxylase (converting Pen N to DAC) and DAC acetyltransferase (converting DAC to CPC) activities were present in the disrupted strain as well as in the others, because there was *in vitro* CPC synthesis. This CPC synthesis was higher in the TDM-139 than in the parental and the complemented strains, probably due to the PenN accumulation in the *cefM* disrupted strain. The endogenous PenN is converted to CPC in the cell-free extracts, when the compartmentalized intermediates are released.

Transformants containing functional *cefM-GFP* fusions produce cephalosporin

Since the bioinformatic analysis showed that *cefM* may interact with peroxin Pex19, the subcellular location of the CefM protein in *A. chrysogenum* was studied *in vivo* by using GFP as reporter. This experiment required the utilization of one mutant lacking CefM as host strain for the fused *cefM-GFP* gene, in order to be sure that the GFP protein is targeted to the proper location by a functional CefM. For this propose, the TDM-139 mutant (disrupted in the *cefM* gene) was transformed with plasmid *pcefM-GFP* (Fig. 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 the GFP.

The *pcefM-GFP* transformants were tested for cephalosporin production by the agar plug method obtaining two transformants that showed a similar cephalosporin production than *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 *Bam*HI-*Mlu*I and hybridized with a 608 pb *Sal*I probe of the *cefM* gene. Results showed (Fig. 5B) that the probe hybridized with a band of 3.3 kb, as expected (Fig. 5B lanes 1 and 2), indicating that the *cefM-GFP* hybrid gene was integrated in a non-reorganized form. The 7.4 kb hybridization band corresponded to the endogenous hybridizing band in the *cefM* disrupted mutant used as host strain (Fig. 5B; lane 3). The fused CefM-GFP protein in these two transformants is functional since it restored cephalosporin production to the blocked mutant.

440 **The fused CefM-GFP protein remains intact in the integral membrane proteins fraction of a TcefM-GFP transformant**

To exclude the possibility that the GFP might be cleaved from the fused CefM-GFP protein, western analysis (Fig. 5C) of the pellet including integral membrane proteins was carried out using extracts of the *TcefM-GFP36* transformant (Fig. 5C, lane 3) and the untransformed mutant TDM-139 (lane 2) and the parental strain *A. chrysogenum* C10 (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 (Fig. 5C, lane 3), as expected, whereas a control sample containing free GFP (Roche Diagnostics Corp., Indianapolis, IN, U.S.A. Fig. 5C, lane 4) showed a molecular mass of 27 kDa. No GFP was observed in untransformed strains (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 (Fig. 6). The parental and the complemented strains grow and differentiate into typical arthrospores chains that are associated with high cephalosporin production (see discussion) (Fig. 6, panels in column A). However the formation of arthrospores in the disrupted strain TDM-139 was impaired (column B). Comparison of phase-contrast (Fig. 6 column C I), fluorescence images (Fig. 6 C II) and merged images (Fig. 6 C III) showed that the CefM-GFP hybrid protein was targeted to a type of medium-sized microbodies, where they produce punctuated fluorescence as shown in the enlarged photograph of one hypha (Fig. 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 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], deacetoxycephalosporin C 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 to PenN [33] that is catalyzed by the isopenicillin N-CoA synthetase and isopenicillin N-CoA epimerase proteins encoded by the *cefD1* and *cefD2* genes, respectively [7]. The peroxisomal targeting signals (PTS) allow the sorting of peroxisomal matrix proteins. There are two types of peroxisomal targeting signals: the C-terminal PTS-1 with consensus sequence SKL 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 PTS1 peroxisomal targeting signal for peroxisomal localization [42]. CefD1 contains a putative PTS1 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 into penicillin N in cell-free extracts

490 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 increased body of evidence showing that export genes are frequently linked to antibiotic biosynthesis genes [47].

495 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 the membrane proteins involved in secretion of those metabolites have been identified and validated by gene disruption. Particular attention deserves 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.

500 It is clear from the Results of this work that the *cefM* gene located downstream of the *cefD1* gene encoding the proposed isopenicillinyl-CoA ligase of the two component epimerization system [7,33] is essential for cephalosporin biosynthesis. The CefM protein belongs to the MFS superfamily of proteins, particularly to Family 3 (drug efflux proteins), the same family of the CefT protein [15]. MFS transporters are single polypeptide secondary carriers that are capable of transporting small solutes and utilize the transmembrane electrochemical gradient of protons or sodium ions to drive the extrusion of drugs from the cell [48].

510 CefM contains 12 TMS domains and might be located in the membrane of microbodies or other intracellular organelles (Fig. 8). The presence of a consensus Pex19-interaction motif (residues 212-221) in CefM suggests that the CefM protein is likely to be recruited by Pex19 to the microbody membrane. This motif is present in structural peroxisomal or microbody membrane proteins [26,32].

515 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.

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

525 The microbodies that we observed are very similar to the peroxisomes identified in *P. chrysogenum* that contain a PST1 peroxisomal-targeted GFP [34]. However, we do not exclude at this time that the fluorescent microbodies might be a more specific cephalosporin biosynthesis-related type of 'lipid bodies'.

530 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*, Pen N is likely to be transported back into the cytosol, since the three

535 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 isopenicillin-CoA by the CefD1 acyl-CoA ligase. Very long 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 (Fig. 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 feedback inhibited.

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 this article, a mutant disrupted in *cefM* fails to produce cephalosporin although it contains the enzymes for the conversion of PenN to 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.

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745 **FIGURE LEGENDS**

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 hipotetical MFS proteins of *A. clavatus* (XP_001273827), *A. fumigatus* (XP_748433) and *N. fischeri* (XP_001258746). The 12 TMS segments are overlined with a solid bar, and the characteristic motifs (A, B, C, D₂ and G) of the MFS family 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 sequenced of the Pex19p binding site in CefM protein (residues 212-221) is boxed.

755 **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 labeled probes internal to the *cefM* (B) or *ble* (C) genes. Lane M, size markers (Lambda DNA / HindIII digested); lanes 1, transformant TDM-38; 2, TDM-57; 3, TDM-64; 4, TDM-87; 5, TDM-131; 6, TDM-139; 7, TDM153; 8, *A. chrysogenum* C10. Note that transformants TDM-87 and TDM-139 contain an intact pDM construction (lanes 4 and 6). Transformant TDM139 shows the correct gene replacement (lane 6). The size of the hybridization bands (in kb) are indicated on the right.

Figure 3. Growth kinetics and β -lactam production in the parental strain, the *cefM*-disrupted mutant and the complemented transformants.

770 Growth kinetics (A), cephalosporin (B and C) and extracellular penicillin (D) production of *A. chrysogenum* C10, the disrupted mutant TDM-139 and the complemented transformants TCM-27. (E) Intracellular level of penicillin N. The error bars indicate the standard deviations of three independent cultures. In panel B analysis of the total cephalosporin production in the culture broths was made by bioassays using *E. coli* ESS2231 as test strain. Panels C and D show HPLC analysis of the cephalosporin C (C) and extracellular penicillin (IPN+PenN) production (D) in the culture broths. (E) HPLC analysis of the intracellular PenN. The same results were obtained with the TCM-28 and TCM-35 transformants (not plotted to avoid overlapping of the lines).

780 **Figure 4. Complementation *in trans* of the disrupted TDM-139 mutant with pCM.**

(A) Plasmid pCM containing the *cefM* gene. (B) Southern blot hybridization of *EcoRI*-digested genomic DNA from five transformants, untransformed *A. chrysogenum* C10, and the disrupted mutant TDM-139, using as probe the 608 pb *SaI*I fragment internal to the *cefM* gene. Lane M, size markers (Lambda DNA / HindIII digested); lanes 1, TCM-9; 2, TCM-10; 3, TCM-27 4, TCM-28; 5, TCM-35; 6, TDM-139 (disrupted mutant); 7, *A. chrysogenum* C10. The size of the hybridization bands (in kb) are indicated on the right.

790 **Figure 5. Southern and western blot analysis of the transformant carrying the fused *cefM*-GFP gene.**

(A) Plasmid *pcefM-GFP* containing the *cefM*-GFP hybrid gene. (B) Southern blot hybridization of *MluI*-*Bam*HI digested genomic DNA from transformants *TcefM-GFP36* (lane 1) and *TcefM-GFP37* (lane 2) transformants, the wild type C10 (lane 3)

795 and TDM-139 (lane 4) using as probe the 608 pb *Sa*II fragment internal to *cefM*. (C)
Immunoblot analysis of the CefM-GFP fusion protein in the membrane fraction of the
transformant *TcefM-GFP36*. Lane M, Bio-Rad Precision Protein Standards; Lanes 1,
TDM-139; 2, C10; 3, *TcefM-GFP36* and 4, free GFP. Immunodetection was performed
800 with anti-GFP antibodies (see Experimental Procedures). The sizes of the hybridization
bands are indicated on the right.

Figure 6. Subcellular localization of the CefM protein.

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

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**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.

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

ACV, δ -L- α -aminoadipyl-L-cysteinyl-D-valine. IPN, isopenicillin N; PenN, penicillin
N; DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; CPC,
820 cephalosporin C.

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

Strain	Genotype	Cephalosporin C production ng CPC/mg of mycelia
<i>A. chrysogenum</i> C10	wild type	7.2 ± 0.8*
<i>A. chrysogenum</i> TDM-139	<i>cefM</i> ⁻	17.8 ± 2.0
<i>A. chrysogenum</i> TCM-27	wild type	7.1 ± 0.5

The strains were grown in DP medium and mycelia were collected at 144 hours. Data are the average of three determinations. * Standard deviation of the mean values.

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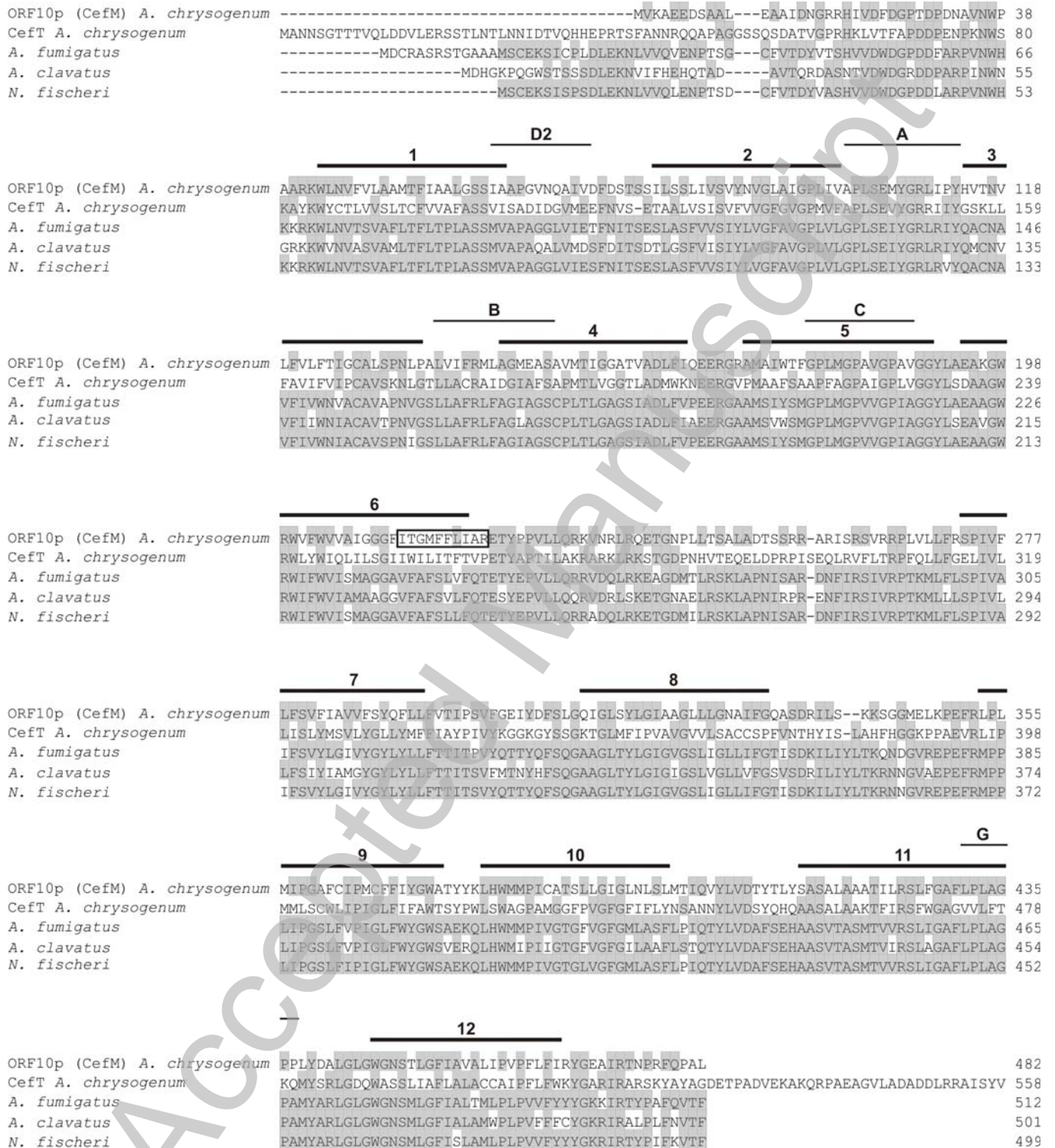
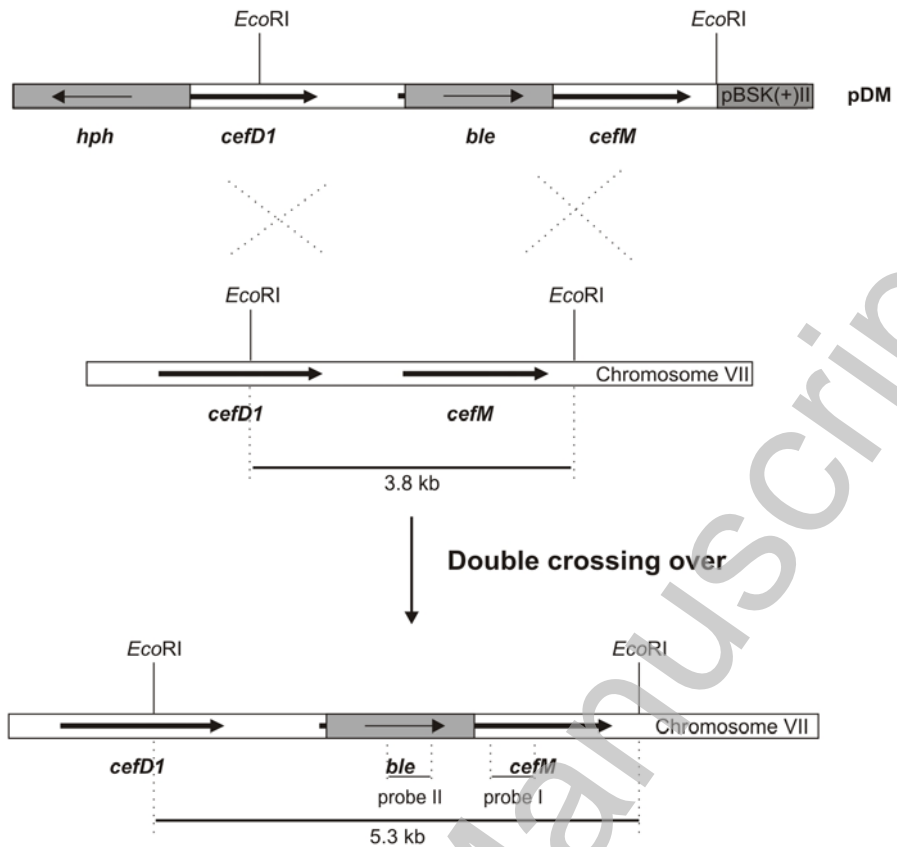


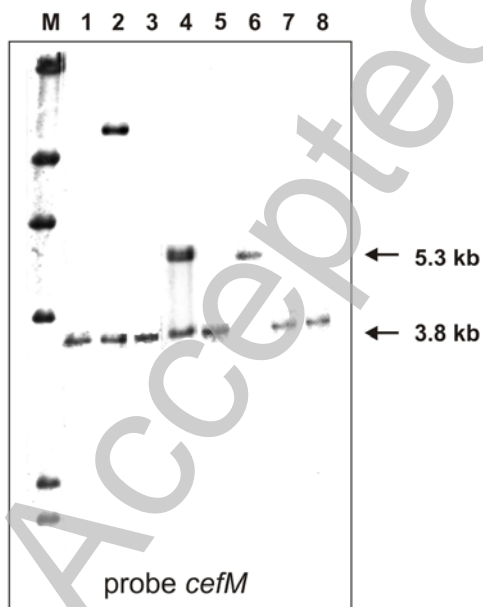
Figure 1. Teixeira et al.

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20081180

A



B



C

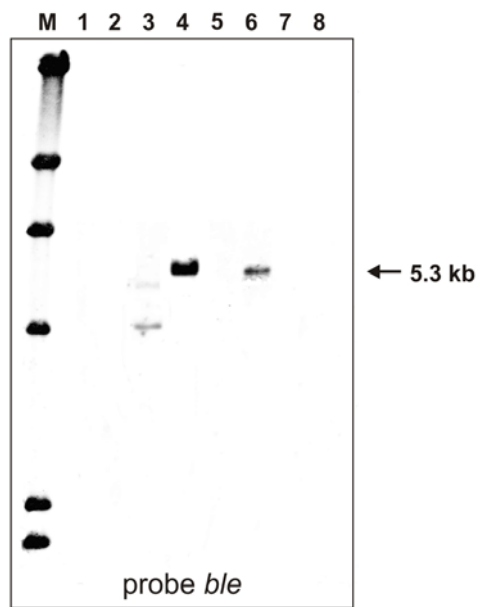


Figure 2. Teixeira et al.

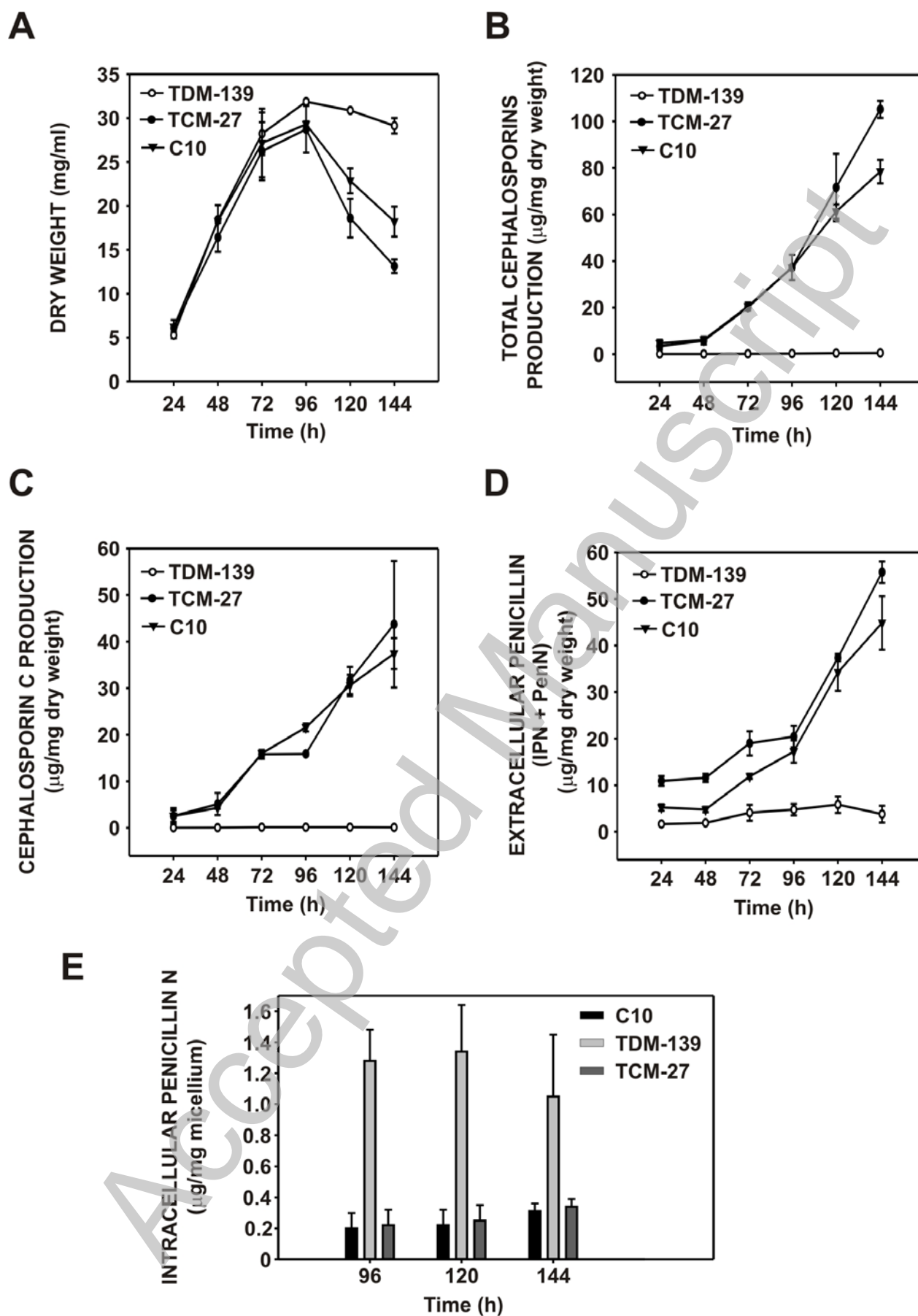
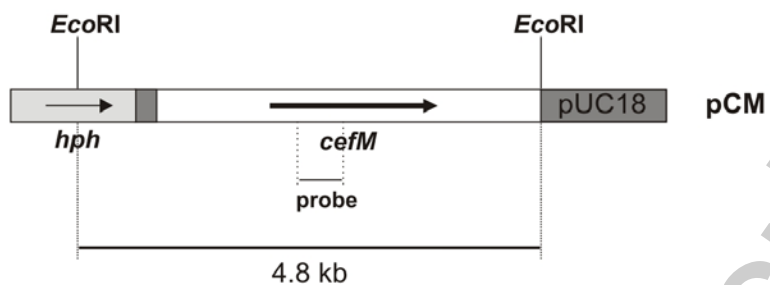


Figure 3. Teixeira et al.

A



B

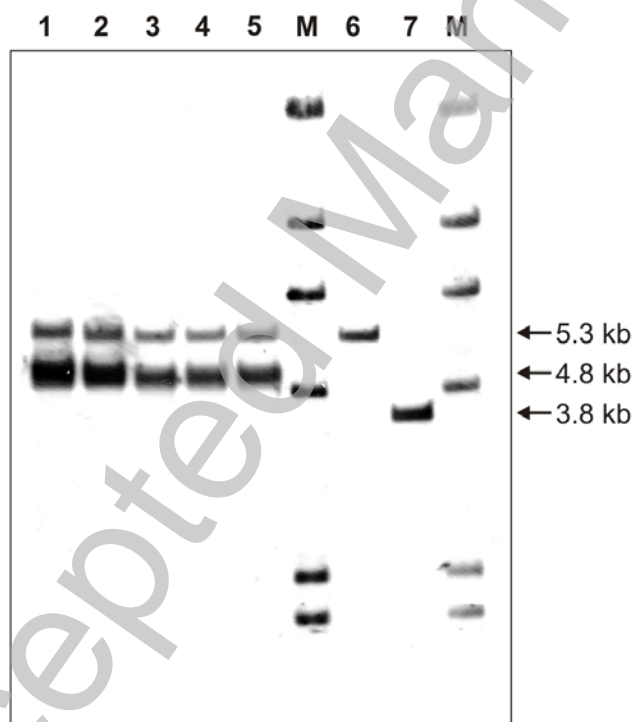


Figure 4. Teixeira et al.

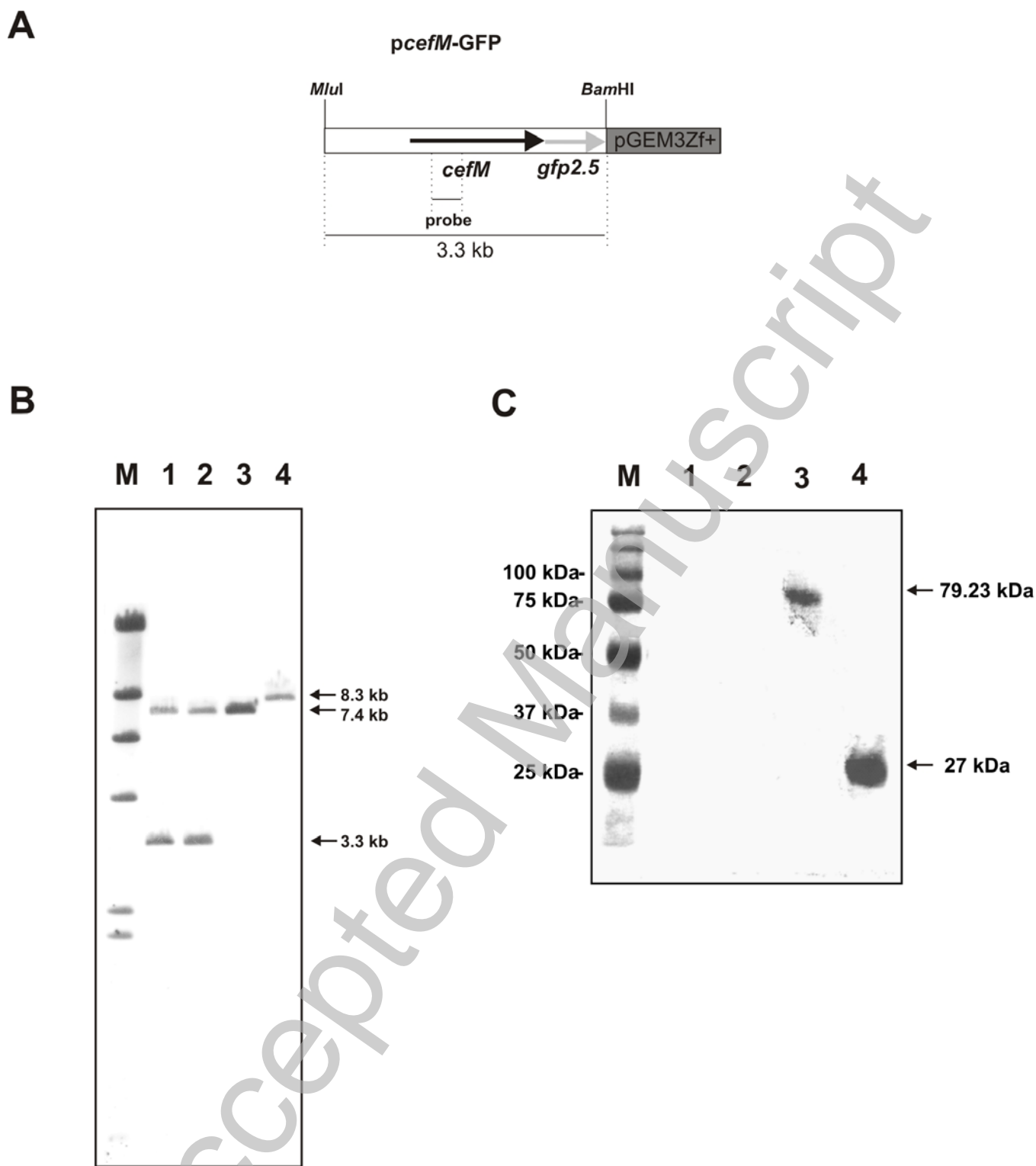


Figure 5. Teixeira et al.

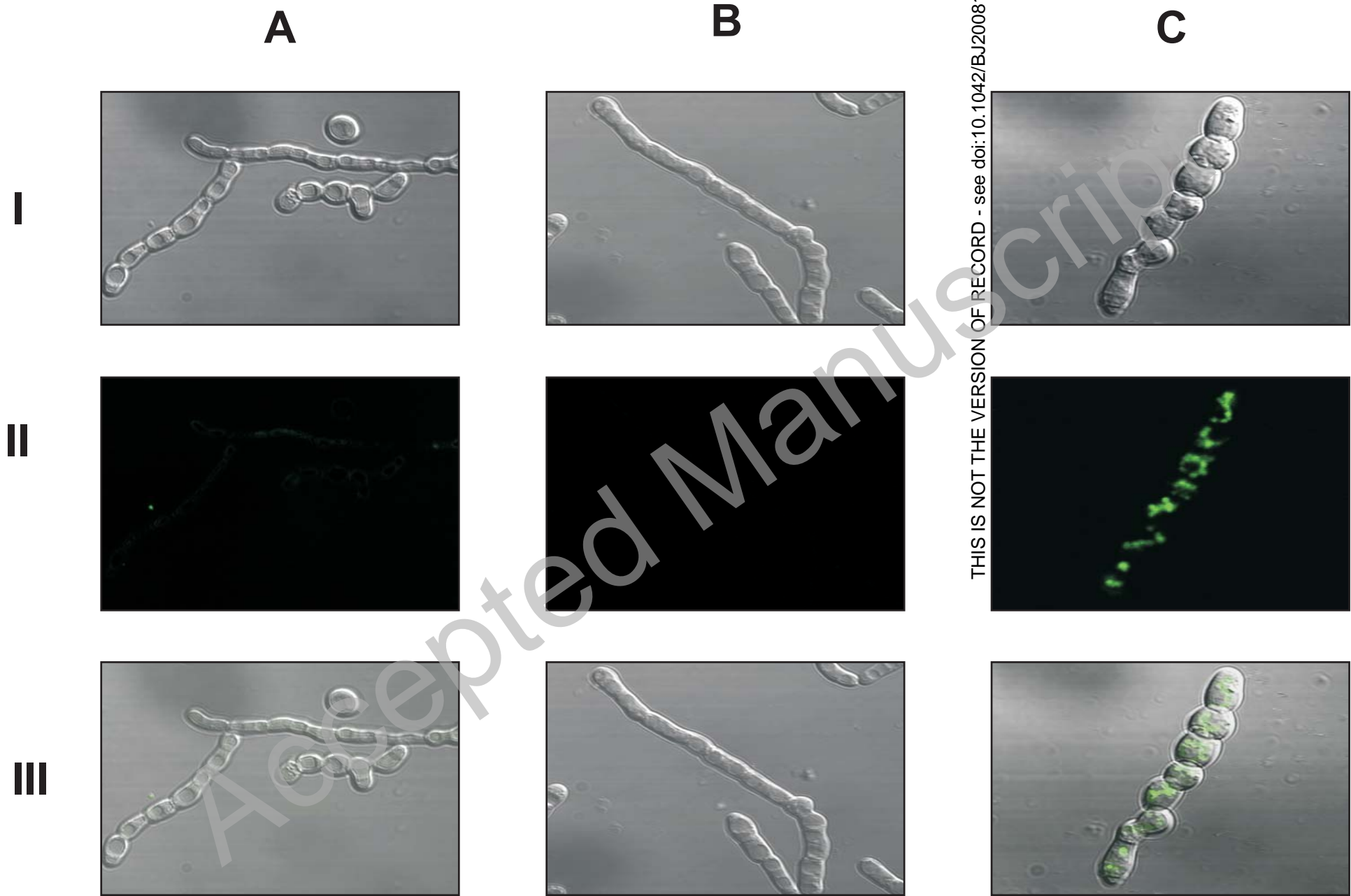


Figure 6. Teijeira et al.

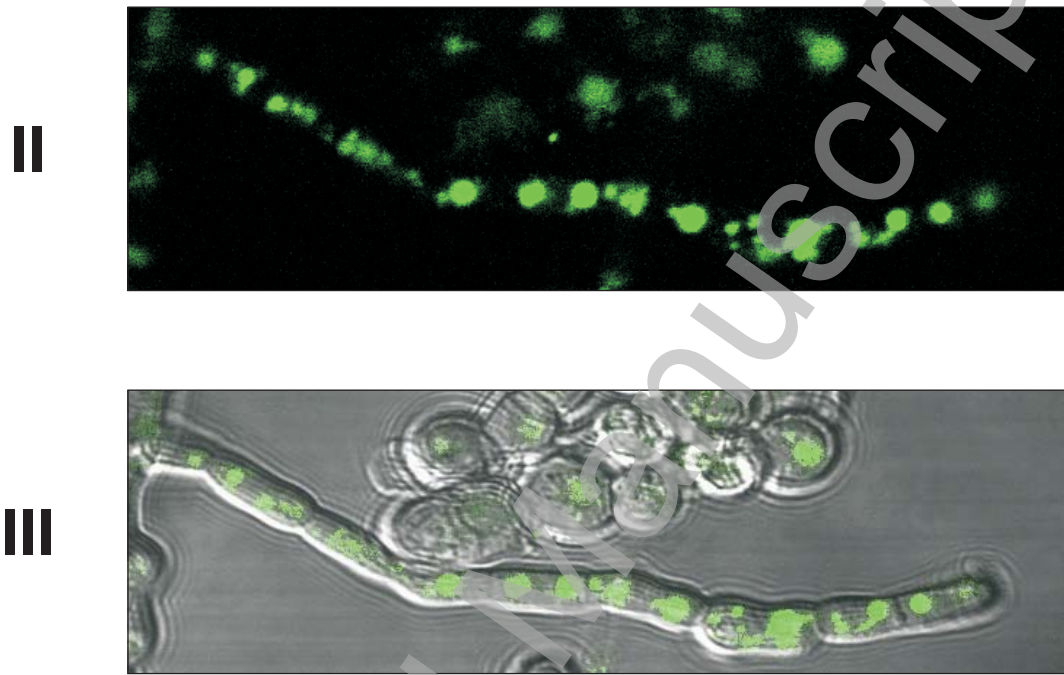


Figure 7. Teijeira et al.

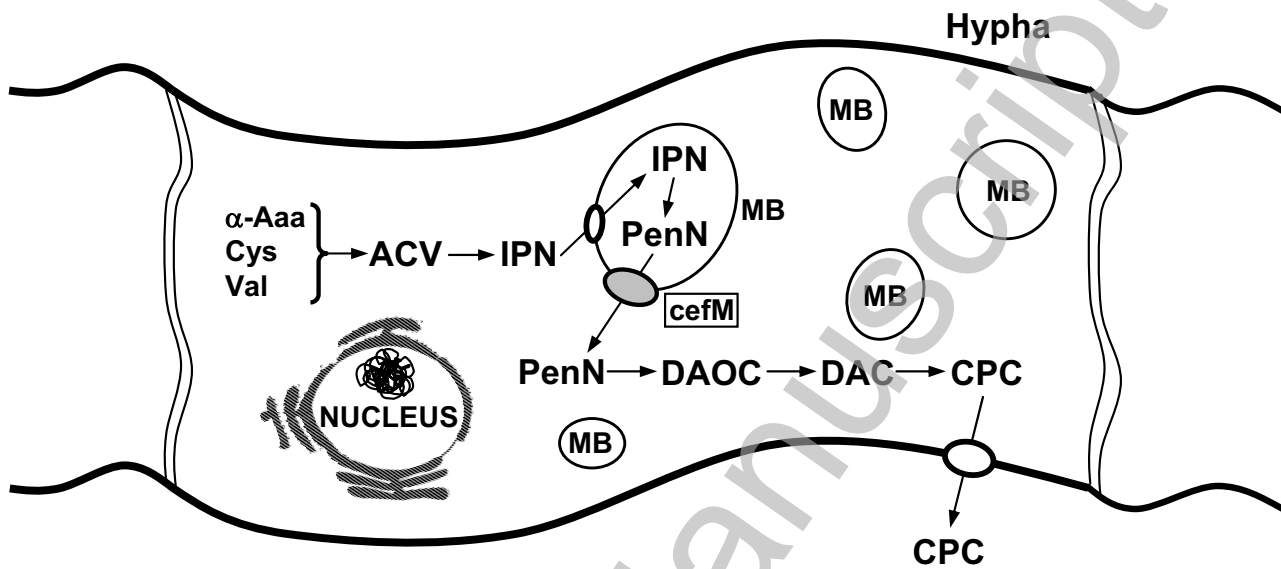


Figure 8. Teijeira et al.