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Abbreviations used: ACV, δ-L-α-aminoadipyl-L-cysteinyl-D-valine; IPN, isopenicillin N; PenN, penicillin N; DAC, deacetylcephalosporin C; CPC, cephalosporin C; cephalosporins, CPC and DAC; LPE, Le Page and Campbell; DP, defined production; ORF, open reading frame; Pex19, peroxisome biogenesis factor 19; PTS, peroxisomal targeting signal; MFS, major facilitator superfamily; GFP, green fluorescent protein; DsRed, red fluorescent protein; RT, reverse transcription; TMSs; TransMembrane Spanners; TDP, transformant disrupted in *cefP*; TCP, transformant complemented in *cefP*; TCR, transformant complemented in *cefR*; TCRP, transformant complemented in *cefR* and *cefP*

ABSTRACT

The mechanisms of compartmentalization of intermediates and secretion of penicillins and cephalosporins in β-lactam producing fungi are of great interest. In *Acremonium chrysogenum* there is a compartmentalization of the central steps of the cephalosporin C biosynthetic pathway. In this work, we found in the 'early' cephalosporin C cluster a new gene named *cefP* encoding a putative transmembrane protein containing eleven transmembrane spanners. Targeted inactivation of *cefP* by gene replacement showed that it is essential for cephalosporin C biosynthesis. The disrupted mutant is unable to synthesize cephalosporins and secretes a significant amount of isopenicillin N indicating that the mutant is blocked in the conversion of isopenicillin N to penicillin N. The production of cephalosporin in the disrupted mutant was restored by transformation with both the *cefP* and *cefR* genes (a regulatory gene located upstream of *cefP* gene) but not with the *cefP* gene alone. Fluorescent microscopy studies with an eGFP-SKL protein (peroxisomal-targeted marker) as control showed that the red fluorescent CefP protein colocalized in the peroxisomes with the control peroxisomal protein. In summary, CefP is a peroxisomal membrane protein probably involved in the import of isopenicillin N into the peroxisomes where it is converted to penicillin N by the two-component CefD1-CefD2 protein system.

INTRODUCTION

Very little is known about the subcellular localization of enzymes involved in the biosynthesis of secondary metabolites in fungi [1,2]. *Acremonium chrysogenum* is a filamentous fungus used for industrial production of β-lactam antibiotics that contains the cephem nucleus. Cephalosporin C biosynthesis begins with the condensation of the three precursor amino acids L-α-aminoadipic acid, L-cysteine and L-valine to form the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) [3,4]. This reaction is carried out by the ACV synthetase [5] which is encoded by the *pcb*AB gene [6]. Later, oxidative ring closure of the ACV tripeptide by the IPN synthase encoded by the *pcb*C gene [7] leads to formation of a bicyclic ring constituted by the four-membered β-lactam ring fused to the five-membered thiazolidine ring. The resulting compound isopenicillin N (IPN), is the first compound in the biosynthetic pathway with antibiotic activity.

The conversion of IPN into penicillin N (PenN) in *A. chrysogenum* involves the activity of an IPN-CoA synthetase encoded by the *cefD1* gene and an IPN-CoA epimerase encoded by the *cefD2* gene [8]. Both enzymes catalyse the isomerisation of the L-α-AAA side chain of IPN to the D-enantiomer to give PenN.

After the epimerization step, a bi-functional enzyme with expandase and hydroxylase activities (encoded by the *cefEF* gene) [9] converts PenN into deacetoxycephalosporin C (DAOC) and then into deacetylcephalosporin C (DAC). As a result, the five-membered thiazolidine ring of penicillin is replaced by a six-membered dihydrothiazine ring forming the cephem nucleus. Finally, DAC is converted into cephalosporin C (CPC) by the DAC-acetyltransferase that uses acetyl-CoA as donor of the acetyl group, encoded by the *cefG* gene [10].

The CPC biosynthetic genes are located in two clusters; *pcbAB*, *pcbC*, *cefD1* and *cefD2* are located in the so-called “early” cluster, while *cefEF* and *cefG* genes are located in the “late” cluster [8,10,11].

While in *Penicillium chrysogenum* there is a compartmentalization of the penicillin biosynthetic pathway [reviewed in 2] between the cytosol and the peroxisomal lumen, in *A. chrysogenum* previous works proposed that probably all CPC biosynthetic enzymes are cytosolic [12,13]. However, the amino acid sequences of the two-component IPN epimerase system (CefD1 and CefD2) contain PTS1 peroxisomal targeting sequences suggesting that the epimerization step takes place in the peroxisomal matrix [2].

The distinct subcellular localization of the β-lactam biosynthetic enzymes in both filamentous fungi implies transport of enzymes, precursors, intermediates and products through these compartments. However, although a good biochemical and genetic knowledge has accumulated about penicillin and CPC biosynthesis [1,2], there is not enough knowledge about the systems involved in such transport.

Recently our group has characterized the *cefM* gene of *A. chrysogenum* that encodes a membrane protein of the Major Facilitator Superfamily (MFS) that is located in small-sized microbodies (probably peroxisomes). The CefM protein seems to be involved in the translocation of the intermediate PenN from the peroxisomal lumen to the cytosol [14].

The question of how IPN is transported from the cytosol to the peroxisomal matrix remains unanswered. In order to search for proteins involved in IPN transport, we

studied the DNA region located upstream of the *cefT* gene [15]. In this article, we report the characterization of a new gene encoding a membrane protein essential for CPC biosynthesis located in the cluster of early CPC genes. We provide evidence for its localization in peroxisomes and propose a role in IPN transport across the peroxisomal membrane.

EXPERIMENTAL

Microorganisms, culture media and antibiotic determination

Acremonium chrysogenum C10 (ATCC 48272) a high-CPC-producing strain provided by PanLabs Laboratories was used as the parental strain in this study. All other strains were derived from *A. chrysogenum* C10; for sporulation they were grown in LPE medium [16] for seven days at 28 ºC. Spores and mycelium fragments collected from six plates of LPE culture medium were inoculated in 100 mL of seed medium [17] in 500 mL shake-flasks and incubated at 25 ºC for 48 h in an orbital incubator at 250 rpm. Ten mL of this seed culture were used to inoculate 100 mL of defined production (DP) medium [17] in triple-baffled flasks (500 mL; Bellco) and incubated at 25 ºC in a rotary shaker (250 rpm). Samples were taken every 24 h. β–lactam antibiotic production was assayed against *E. coli* ESS2231 (a β-lactam supersensitive test strain) and determined by HPLC as described previously by Ullán et al. [18].

DNA isolation and Southern blotting

Genomic DNA of *A. chrysogenum* was isolated as described previously [19]. Genomic DNA samples (3 μg) from *A. chrysogenum* C10 and its transformants were digested with restriction enzymes and separated in 0.7 % agarose gels. Digested total DNA was separated by agarose gel electrophoresis and blotted onto nylon membranes (Hybond NX, Amersham Pharmacia Biotech) [20]. Southern blot hybridization was performed as previously described [21].

RNA isolation

Total RNA was isolated from *A. chrysogenum* C10 mycelia with the RNeasy kit (Quiagen) as described previously [21].

DNA sequencing and intron analysis

Sequencing reactions of the DNA were made by standard procedures [20] and automatic sequencing was performed with the AutoReadTM System (Pharmacia, Uppsala, Sweden). To elucidate the presence of putative introns in the DNA sequence of the *cefP* gene, the DNA region containing the expected intron splicing sites was amplified by RT-PCR (Promega) using RNA of *A. chrysogenum* 48 h cultures as template with the primer pairs:

INT-CP-DIR: 5´-GCGAATGCGACCCCGAGGAGTA-3´
INT-CP-REV: 5´-TCGCAACAAAGAAGTAGGTGAAGA-3´
INT-CP(B)-DIR: 5´-CTACTACTTCGGGCAGCGGTT-3´
INT-CP(B)-REV: 5´-ATGTTATTTTCGTCAGTGTCC-3´

The amplified regions were sequenced to confirm the presence of the 3 introns.

PCR and RT-PCR analysis

Genomic DNA amplification of the *cefR* gene was made by PCR using the oligonucleotides R1R (5´-ATGCAGCCCAGGTTATAA-3´) and RF5 (5´-ATTCATGTCACAAGCCCC-3´) as specific primers.

Expression of the *cefP* and *cefR* genes was tested by RT-PCR using primer pairs INT-CP-DIR/INT-CP-REV and RF6 (5´-ATAGCAGGGATGGCGACAG-3´)/R4R (5´-ACACCATCCGAAAGCACA-3´) respectively.

Site-directed mutagenesis

In vitro mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene) by following the manufacturer's instructions. Oligonucleotides A1 and A2 were used to introduce a *Bgl*II site in the *cefP* gene.

A1: 5´-GATTGGAGAGAGATCTGATGATCTGGTGG-3´

A2: 5´-CCACCAGATCATCAGATCTCTCTCCAATC-3´

Plasmid constructions containing the *cefP* and/or *cefR* genes

pCP. Contains a *Bam*HI fragment of 7.2 kb bearing the *cefP* gene under the control of its own promoter cloned into the *Bam*HI site of plasmid pJL43 [22].

pDP. A *Bgl*II restriction site was introduced by in vitro mutagenesis into plasmid pCP. The *cefP* gene was inactivated by insertion of the hygromycin resistance (*hph*) cassette (subcloned from pAN7-1; [23]) into this *Bgl*II site. Plasmid pDP contains also the phleomycin-resistance (*ble*) gene under the control of the *P. chrysogenum pcbC* promoter as a second selective marker.

pB5.5R. This plasmid carries a *Not*I fragment of 5.3 kb containing the *cefR* gene under the control of its own promoter cloned into the *Not*I site of the pBluescript II SK$^+$ (Stratagene).

pCRP. This plasmid contains a *Bam*HI fragment of 8.5 kb bearing both the *cefP* and *cefR* genes under the control of their own promoters cloned into the *Bam*HI site of the pBluescript II SK$^+$ (Stratagene) plasmid.

pcefP-DsRed. To obtain the fused *cefP-DsRed* gene, a *Bgl*II-*Stu*I 2.7 kb DNA fragment obtained from plasmid pDP by PCR using the ST3-F (5´-GGAAGATCTATGTTCGGATCTAGAGATGGC-3´) and T3DR-R (5´-AAAAGGCCTAATGTTATTTTCGTCAGTGTC-3´) oligonucleotides was inserted into the *Bgl*II-*Sma*I site of pEXpDsRed bearing the *DsRed* gene from *Discosoma* sp. (Clontech) flanked by the *gdh* gene promoter from *A. nidulans* and the terminator of the *cyc1* gene from *Saccharomyces cerevisiae*.

p43EFGP-SKL. This plasmid contains the eGFP-SKL gene which encodes a protein targeted to peroxisomes. To construct p43EFGP-SKL a *Not*I 2.1 kb fragment from pGBRH2-eGFP-SKL (1) was inserted into the *Not*I site of pJL43.

Transformation of *A. chrysogenum* protoplast

A. chrysogenum protoplasts were obtained and transformed as previously described [19]. Transformants were selected in TSA (tryptic soy agar, Difco) with sucrose (10.3 %) supplemented with phleomycin (10 μg/mL) or hygromycin B (30 μg/mL).

cefP gene disruption

Plasmid pDP was transformed into *A. chrysogenum* C10, and transformants were selected by their hygromycin B resistance. Transformants showing a hyg^R $phle^S$ phenotype, indicating that double recombination had occurred, were selected.

Integration of the hybrid *cefP-DsRed* and subcellular localization of the hybrid protein

Protoplasts of *A. chrysogenum* C10 were transformed with the integrative plasmids pPDsRed and p43EGFP-SKL (the latter is a derivative of pGBRH2-eGFP-SKL; [1]). Plasmid pPDsRed carries the hybrid gene *cefP-DsRed* under the control of the promoter of the *A. nidulans gdh* gene and the terminator of the *cyc1* gene of *S. cerevisiae*. On the other hand, plasmid p43EGFP-SKL carries the *eGFP* gene coupled with a SKL sequence (for its targeting to peroxisomes) under the control of the *pcbC* gene promoter and the *penDE* gene terminator of *P. chrysogenum*. In addition, this plasmid carries the phleomycin resistance cassette for transformant selection.

For the subcellular localization of the hybrid protein CefP-DsRed, spores of transformant TPDsRed-32 were inoculated in CCM (complex culture medium) [14] and incubated for 3 days at 25 ºC and 175 rpm for its adequate germination and growth. This seed culture was used to inoculate DP medium (10 % inoculum) and incubated for 72 h at 25 ºC and 250 rpm.

Fluorescence microscopy

The fluorescence emissions of hyphae were analysed by confocal laser-scanning microscopy using a Radiance 2000 laser confocal microscope (Bio-Rad Laboratories). Green (eGFP) and Red (DsRed) fluorescent proteins were visualized with a number 13 filter (470 nm excitation, 20 nm bandwidth; 505–530 nm emission for GFP protein and 556 nm excitation, 20 nm bandwidth; 586 nm emission for DsRed protein).

RESULTS

Identification of the *cefP* gene downstream of *cefT*

A *Sma*I fragment (4.6 kb) of the DNA region located downstream of the *cefT* gene (Supplementary figure 2) was cloned into the pBluescript KS (+) plasmid in both orientations, giving rise to plasmids pP1a and pP1b. The inserts of pP1a and pP1b were completely sequenced on both strands. The nucleotide sequence was deposited in the EMBL database under the Accession number AM231816 (*cefP*). Analysis of the nucleotide sequence of the 4.6 kb DNA insert revealed the presence of one ORF named *cefP* (for peroxisomal protein; see below). The *cefP* gene is 2769 nt long and is interrupted by the presence of three introns. The presence of the introns was confirmed

by RT-PCR as described in Experimental Procedures. The *cefP* gene encodes a protein of 866 amino acids with a deduced molecular mass of 99.2 kDa. The amino acid sequence of the CefP protein showed strong similarity throughout its entire length to uncharacterized integral membrane proteins (Supplementary figure 1) of *Nectria haematococca* (63 % identical amino acids), *Gibberella zeae* (60 % identical amino acids), *Podospora anserina* (52 % identical amino acids), *Chaetomium globosum* (50 % identical amino acids), *Neurospora crassa* (49 % identical amino acids) and *Coccidioides immitis* (41 % identical amino acids). Furthermore, we found in all proteins one DUF221 [pfam02714] motif that occurs a superfamily of hypothetical transmembrane proteins none of which have any known function (http://pfam.ccbb.re.kr/cgi-bin/getdesc?name=DUF221)

Interestingly, there were no proteins with high similarity in the genomes of the benzylpenicillin producers *Penicillium chrysogenum* or *Aspergillus nidulans* (which do not produce cephalosporins), suggesting that this protein is likely to be specific for CPC biosynthesis.

To determine the number of TransMembrane Spanners (TMSs) the deduced CefP protein was analyzed with the algorithms of five different programs: SOSUI [24] (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html), TopPred2 [25] (http://bioweb.pasteur.fr/seganal/interfaces/toppred.html), TMHMM [26] (http://www.cbs.dtu.dk/services/TMHMM/), TMPRED [27] http://www.ch.embnet.org/software/TMPRED_form.html) and HMMTOP [28] (http://www.enzim.hu/hmmtop/html/submit.html). Results showed nine putative TMSs with SOUSI and TopPred2 bioinformatic tools whereas TMPRED algorithm predicted nine or ten TMSs. Nevertheless, TMHMM and HMMTOP programs increased the number of putative TMSs to eleven. The evaluation of these methods by Möller et al. [29] revealed that the best algorithms are those of the HMMTOP and TMHMM programs. In summary CefP protein contains from nine to eleven TMSs being eleven the most probable number (Supplementary figure 1 shows the location of every TMS). The number of amino acids in each TMS ranges from 17 to 23.

Analysis of Pex19 (peroxisome biogenesis factor 19) binding sequences (http://www.peroxisomedb.org/) in the CefP protein revealed one putative Pex19 binding site [30] between the amino acids 460 and 469 (See supplementary figure 1). These observations suggested that CefP may be a peroxisomal membrane protein [31] (see below).

Targeted inactivation of *cefP* results in isopenicillin N accumulation

To determine if the CefP protein is involved in CPC biosynthesis, we inactivated the *cefP* gene by the double marker technique [8,15,32,33]. Targeted inactivation of this gene was performed using plasmid pDP (see Experimental). This plasmid carries an inactivated *cefP* gene [obtained by insertion of the hygromycin B resistance gene (*hph*) under the control of the *gpd* gene promoter, as transformation marker]. To confirm that targeted inactivation took place at the right position, five transformants and the *A. chrysogenum* C10 parental strain (positive control), were analyzed by Southern blot. The DNA of all strains was digested with *Sma*I and hybridized with a 4.6 kb *Sma*I probe

containing the *cefP* gene (Supplementary figure 2A). Results showed that the control strain *A. chrysogenum* C10 (Supplementary figure 2B, lane 6) hybridized with a genomic DNA band of 4.6 kb; however, in TDP (for transformants disrupted in *cefP*) transformants [TDP-115 (Supplementary figure 2B, lane 1), TDP-151 (Supplementary figure 2B, lane 2) and TDP-206 (Supplementary figure 2B, lane 3)] the 4.6 kb hybridization band was converted into two bands of 7.5 kb and 1.1 kb, as expected, by a canonical double recombination (Supplementary figure 2A).

To confirm that the *cefD1-cefD2* and *cefEF-cefG* genes were not disrupted during the *A. chrysogenum* transformation process, a Southern blot analysis was performed. The DNA of the three strains was digested with *Bam*HI and hybridized with one probe of the *cefD1-cefD2* bidirectional promoter region (1 kb *Hind*III-*Eco*RV, Supplementary figure 2C) and another probe of the *cefEF-cefG* genes (7.3 kb *Bam*HI, Supplementary figure 2C). Results showed that the *A. chrysogenum* C10 (Supplementary figure 2D, lane 4) and the transformants TDP-115 (Supplementary figure 2D, lane 1) and TDP-206 (Supplementary figure 2D, lane 3) hybridized with two genomic DNA bands of 14 kb (*cefD1-cefD2*) and 7.2 kb (*cefEF-cefG*) as expected (Supplementary figure 1C). However, in the transformant TDP-151 the 14 kb hybridization band was converted into a band of 11 kb suggesting a reorganization of the *cefD1-cefD2* locus probably by an integration of the pDP plasmid in the *cefD1-cefD2* locus (Supplementary figure 2D, lane 2). This transformant was therefore discarded.

The two *cefP* disrupted transformants (TDP-115, TDP-206) and *A. chrysogenum* C10 as control strain were cultured in DP medium. In TDP transformants a drastic reduction of cephalosporins production (DAC and CPC) was detected by bioassay (about 8 % of the cephalosporins produced in *A. chrysogenum* C10) whereas in the same culture conditions, a high cephalosporins production was observed in *A. chrysogenum* C10 (Figure 1A). Analysis by HPLC of the culture broth supernatant (Figures 2B and C) confirmed the bioassay's results and showed that the CPC (Figure 1B) and DAC (Figure 1C) production in the disrupted strains were drastically reduced. However, there was an increment in the penicillin (IPN and PenN) production (Figure 1D). Further HPLC analysis of the late culture broths (96 h, 120 h and 144 h) of the disrupted strains indicated that the penicillin in this mixture was IPN whereas in the parental strain only PenN was detected (Figure 1E).

To study whether the protein encoded by the *cefP* gene was involved in IPN transport to peroxisomes, the intracellular cephalosporins levels (DAC and CPC) were measured by HPLC [18] in cell extracts of the disrupted TDP transformants and the parental strain *A. chrysogenum* C10 grown in DP medium for 96, 120 and 144 h. Results showed, when compared to the parental strain, an inefficient biosynthesis of DAC (Figure 2A) and the lack of CPC formation (Figure 2B) in the disrupted mutants. Additionally, there was no penicillin N accumulation in *cefP*-disruptant strains (see below).

Taken together, these results indicate that the TDP strains are unable to convert IPN to PenN and cephalosporins in an efficient manner, probably due to a lack of IPN transport related to its conversion into PenN and DAC and, as a result, an increased amount of extracellular IPN is accumulated.

Cell free extracts allow in vitro CPC synthesis

All other genes of the CPC pathway appeared to be functional in the *cefP*-disrupted strains (Supplementary figure 2 D, lane 1 and 3). This was confirmed by *in vitro* conversion of IPN to CPC using cell-free extracts of the transformant TDP-115 (disrupted in *cefP*) and the parental strain grown in DP medium for 144 h as described previously by Teijeira et al. [14]. Results showed (Table 1) that the IPN-CoA synthetase/IPN-CoA epimerase system (converting IPN to PenN), the expandase/hydroxylase (converting PenN to DAC) and DAC acetyltransferase (converting DAC into CPC) activities were present in the disrupted mutant as well as in the parental strain, because there was *in vitro* CPC synthesis. The IPN is converted to CPC in the cell-free extract, when the compartmentalized CefD1 and CefD2 enzymes are released.

Complementation of the *cefP* mutation requires additional genetic information

For complementation studies plasmid pCP, bearing the intact *cefP* gene with its own promoter (Figure 3A), was transformed into the TDP-206 and TDP-115 strains and transformants were selected by their resistance to phleomycin. Total DNA was extracted from the TDP-206 and TDP-115 blocked mutants (from three transformants of each disrupted strain) and from the parental strain *A. chrysogenum* C10; the DNAs were digested with *Sma*I and hybridized with a 4.6 kb *Sma*I probe containing the *cefP* gene. Results showed (Figure 3A) that the probe hybridized with a band of 4.6 kb, as expected (Figure 3A, lanes 1 to 6), indicating that the *cefP* gene was integrated in a non-reorganized form. The 7.5 kb and 1.1 kb hybridization bands corresponded to the endogenous hybridizing DNA-fragments in the *cefP* disrupted mutants (Figure 3A; lanes 7 and 8) used as host strains.

The effect of complementation of *cefP* on CPC production was studied with two different transformants named TCP (for transformants complemented in *cefP*). Results showed that in transformants TCP-105 (obtained from TDP-206 complemented with the *cefP* gene) or TCP-12 (derived from TDP-115 complemented with the *cefP* gene), the cephalosporins production (Figure 3B) was not restored. To confirm these results, three hundred transformants (150 of each disrupted strain) that showed resistance to phleomycin were tested for cephalosporin production by the agar plug method [18].The results showed again that in none of the *cefP*-complemented transformants the cephalosporins production was restored (data not shown).

There is no expression of the *cefP* gene in the TCP strains

To study if the *cefP* gene is really expressed in the complemented TCP strains we analysed its expression by RT-PCR using the primers indicated in the Experimental section. The RT-PCR results (Figure 3C) indicated that the *A. chrysogenum cefP* gene is expressed in the control *A. chrysogenum* C10 (Figure 3C, lane 1), but not in the disruptant strain TDP-115 (Figure 3C, lane 2) or in the complemented TCP-12 strain (Figure 3C, lane 3). Upstream of the *cefP* gene there is a gene named *cefR* involved in *cef* genes regulation (Teijeira F, Ullán RV and Martin JF; unpublished results). To clarify

the lack of complementation, the expression of the *cefR* gene was analyzed by RT-PCR in the parental strain, the TDP-115 disrupted transformant and the complemented strain TCP-12 (Figure 3D). Surprisingly, results showed that there is no expression of *cefR* in the disrupted TDP-115 (Figure 3D, lane 2) and in the complemented TCP-12 strains (Figure 3D, lane 3). To confirm that the *cefR* gene is not altered in the TDP-115 strain as a result of the transformation process we amplified this gene by PCR using total genomic DNA of this strain as template. The primers used (see Experimental) are located on both sides of the *cefR* gene and amplified a DNA product of 3.3 kb. This DNA fragment was cloned and sequenced confirming that there is not any mutation in the *cefR* gene or in its promoter region.

Complementation with both *cefP* and *cefR* genes is required for restoration of cephalosporins production

To elucidate if the *cefP* and *cefR* genes were both or just *cefR* necessary to restore the CPC synthesis in the *cefP*-disruptant strain, two series of strains transformed with either *cefR* alone or with both *cefP* and *cefR* were created. For this purpose, we constructed plasmids pB5.5R [bearing only the *cefR* gene (Supplementary figure 3B)] and pCRP [bearing both the *cefR* and *cefP* genes (Supplementary figure 3B)]. These plasmids were cotransformed with the help of pJL43 (22) in the TDP-115 mutant and transformants were selected by resistance to phleomycin. We selected at random one transformant of each transformation, TCR-45 (complemented with *cefR* gene) and TCPR-27 (complemented with *cefR-cefP* genes), which were analyzed by Southern blot hybridization to confirm the correct gene integration (Supplementary figure 3 D and E). For this analysis, TCR-45, TDP-115 and C10 genomic DNAs were digested with *Sma*I and hybridized with a 759 bp *EcoR*I internal fragment of *cefR* as probe (Supplementary figure 3B). Results showed (Supplementary figure 3D) that transformant TCR-45 (Supplementary figure 3D, lane 3) gave hybridization with a DNA band of 4.2 kb in addition to the endogenous 6.2 kb hybridization band [corresponding to the endogenous *cefR* gene in the host TDP-115 strain (Supplementary figure 3D, lane 2)] indicating that the insert of the plasmid pCR was not reorganized.

Similarly, transformant TCPR-27 obtained with the pCRP plasmid was selected, and their DNA digested with a mixture of *Bam*HI and *Fsp*I and hybridized with the *cefP* probe (Supplementary figure 2A). Results showed (Supplementary figure 3E, lane 2) a correct integration of *cefP-cefR* genes, as expected (Supplementary figure 3A); because there was a hybridization band of 8.5 kb [like in the C10 control (Supplementary figure 3E, lane 3)] in addition to the endogenous 12.5 kb corresponding to the disrupted *cefP* gene that also appears in the TDP-115 strain (Supplementary figure 3E, lane 1).

In summary, we obtained three types of complemented strains (Table 2) TCP-12 (complemented with the *cefP* gene), TCR-45 (complemented with the *cefR* gene) and TCPR-27 (complemented with both *cefP* and *cefR* genes). Analysis of the cephalosporins production of each of these strains by bioassay (Figure 4A and B) revealed that only in the *cefP-cefR* complemented strain (TCPR-27) there was an efficient cephalosporin biosynthesis. HPLC analysis of the culture broths confirmed the bioassay results and showed that in transformant TCPR-27 the CPC and DAC

production was restored to levels similar to those of *A. chrysogenum* C10 (Figure 4 C and D). In transformants TCR-45 and TCP-12 carrying only one complementing gene (either *cefR* or *cefP*) (Figure 4 C and D) only a very small amount of cephalosporins was detected as in the disrupted TDP-115 strain.

Analysis of extracellular penicillin levels by HPLC (Figure 4E) showed that the penicillin production decreased to normal parental levels in the TCPR-27 transformant with respect to the disrupted strain (47 % reduction) whereas TCR-45 and TCP-12 strains showed less reduction (33 %) than the *cefP-cefR* complemented strain. Analysis of the penicillin isomers (IPN and PenN) in the late culture broth supernatants (96 h, 120 h and 144 h) revealed that transformant TCPR-27 secretes PenN in similar levels to the parental strain *A. chrysogenum* C10 whereas in the culture broth of the strains complemented with a single gene the only penicillin found was IPN (Figure 4F). Intracellular penicillin analysis of the strains (Figure 4G) showed that the intracellular penicillin detected in the *cefP-cefR* complemented strain (TCPR-27) was PenN. In addition, RT-PCR analysis of the complemented TCPR-27 strain showed expression of *cefP* (Figure 3C, lane 4) and *cefR* (Figure 3D, lane 4) genes.

In summary, integration of both *cefR* and *cefP* genes is necessary to restore the cephalosporins production in the *cefP*-disruptant strain (see Discussion).

The CefP protein is localized in peroxisomes

In order to study the subcellular CefP localization, *A. chrysogenum* C10 was transformed with the plasmids containing the eGFP-SKL peroxisomal-targeted fluorescent protein and the CefP-DsRed fusion protein as described in Experimental. The phleomycin-resistant transformants were analyzed by Southern blot. Their DNAs were digested with a mixture of *Apa*I-*Eco*DR2 and hybridized with a probe internal to the *DsRed* gene. Results of the Southern analysis (Supplementary figure 4B) revealed that the transformant TPDsRed-32 (Supplementary figure 4B, lane 9) shows an hybridization band of 4.5 kb corresponding to the *Pgdh-cefP-DsRed*-T*cyc*1 cassette, that is absent in other transformants (Supplementary figure 4B, lanes 2 to 9) or in the parental strain (Supplementary figure 4B, lane 1). Additionally, to confirm the correct integration of the P*pcbC-eGFP-SKL*-T*penDE* cassette in the genome of transformant TPDsRed-32, new hybridization analysis were performed, digesting with *Not*I the DNA of TPDsRed-32 and of the parental *A. chrysogenum* C10 strain followed by hybridization with a probe corresponding to the *eGFP-SKL* gene. Results showed a single 2.1 kb hybridizing band corresponding to the correct integration of the eGFP-SKL expression cassette in the TPDsRed-32 transformant (Supplementary figure 4C, lane 1).

Confocal microscopy analysis of the fluorescence in *A. chrysogenum* cells grown as indicated in Experimental Procedures revealed that the green fluorescent eGFP-SKL protein is located in the peroxisomes (Figure 5A) as expected [1] and the hybrid CefP-DsRed (red fluorescence) was located in the same position (Figure 5B). Superposition of both fluorescences resulted in a yellow color (Figure 5C), confirming that the CefP is a peroxisomal membrane protein.

DISCUSSION

In *A. chrysogenum* the central step of the biosynthetic pathway of CPC is the conversion of IPN into its D-isomer PenN. In CPC-producing fungi, this reaction of epimerization is catalyzed by two enzymes isopenicillinyl N-CoA synthetase and isopenicillin N-CoA epimerase encoded by the *cefD1* and *cefD2* genes respectively [8,34].

Bioinformatic analysis of CefD1 and CefD2 proteins revealed that both proteins contain putative peroxisomal targeting signals (PTSs) characteristic of peroxisomal matrix proteins [35] that indicated a possible peroxisomal matrix localization of these enzymes [2]. CefD2 protein contains putative PST1 [consensus sequence: (R/L)-(L/V/I)-X5-(H/Q)(L/A)] and PST2 [consensus sequence: (S/C/A)-(K/R/H)-L] signals whereas CefD1 contains only a putative PTS1. Moreover the optimum pH for the in vitro IPN epimerization [8] coincides with the estimated for the peroxisomal lumen [36]. Supporting this possible peroxisomal location, the CefD1 and CefD2 homologue proteins of *P. chrysogenum* have been found in the peroxisomal matrix [1]

Therefore, the IPN precursor and PenN product of the epimerization step, that are hydrophilic antibiotics, requires specific transport across the peroxisomal membrane

In the same 'early' CPC cluster we found previously an open reading frame named *cefM*, located downstream from *cefD1* gene in the same orientation encoding an efflux pump protein with 12-TMSs bearing the characteristic motifs of Drug H+ antiporters (Family 3; drug efflux protein) [14]. Targeted inactivation of *cefM* gene affected significantly antibiotic biosynthesis. The *cefM* disrupted mutant showed a drastic reduction in the extracellular PenN and cephalosporins production and accumulated intracellular PenN. In Vivo confocal microscopy study of CefM–GFP fusion demonstrates that the CefM protein is located in the membrane of small sized microbodies where it appears to be implicated in the PenN translocation from the microbody lumen to the cytosol [14]. In conclusion, the epimerization process takes place inside the microbodies (peroxisomes), but it was still unknown which protein is responsible for IPN importation from the cytosol to the peroxisomal matrix.

Based on gene clustering patterns for secondary metabolite biosynthesis [37,38] we found in this work a new open reading frame, named *cefP*, located upstream of the *cefT* gene [15]. Bioinformatic analysis of the CefP protein revealed that this protein has 11 putative TMSs and one DUF221 (pfam02714) motif that is found in a family of hypothetical transmembrane proteins. Computer analysis also revealed the presence of a Pex19p-binding domain (located between amino acids 460 and 469) characteristic of proteins that are recruited by the peroxin Pex19 protein to be incorporated in the peroxisomal membrane [30]. The Pex19 protein acts both as an import receptor and as a cytosolic chaperone for peroxisomal membrane proteins [39].

To study the CefP protein role we have inactivated the *cefP* gene by the double-marker procedure [8,14,15,33]. The cephalosporins production decreased drastically in the disrupted TDP strains indicating that this protein is essential for CPC biosynthesis *in vivo*. The disrupted mutants accumulated IPN in the culture broths due to an overflow of this intermediate when its intracellular level increases. A similar accumulation of IPN

takes place in the *cefD1-cefD2* disrupted mutants [8] indicating that the IPN epimerization into PenN is indeed blocked in TDP mutants. However, unlike what we found in the *cefD1-cefD2* disrupted mutants, the TDP strains constructed in this article contain *in vitro* IPN epimerase activity for the efficient conversion of IPN into PenN. Similar *in vitro* conversion of PenN to CPC using cell-free extracts of the *cefM* disrupted strain showed that the late CPC enzymatic activities were present although *cefM*-null mutants were unable to produce this β-lactam antibiotic [14].

These results indicate that IPN transport across the peroxisomal membrane is blocked as a consequence of the lack of the CefP transporter in the *cefP* disrupted strains explaining the increased secretion of the intermediary IPN to the culture broth. It is known that the CefT protein is involved in the secretion of hydrophilic β-lactams including IPN [40,41]. CefT seems to be responsible for the secretion of the intracellular IPN excess from the cell to the extracellular media in the epimerase-null or in *cefP*-disrupted strains. In the wild type and the improved CPC production strains IPN is secreted in low amounts because in those strains it is efficiently converted to cephalosporins [2,42].

Surprisingly complementation *in trans* with the single *cefP* gene under the control of its own promoter was not sufficient to restore a wild type phenotype in the TDP mutant but requires the simultaneous introduction of *cefR* gene located upstream of the *cefP* in the same orientation. RT-PCR analysis showed that the *cefR* gene is not expressed in the TDP mutants. Apparently, a functional CefP protein is required for induction of *cefR* expression, perhaps through the transport of an inducer molecule. Complementation with the entire *cefR-cefP* fragment (8.5 kb) restores the wild type phenotype indicating that both linked genes form a cluster that is essential and necessary for an efficient β-lactam biosynthesis (F. Teijeira, R.V. Ullán and J.F. Martín, unpublished).

This work confirms the compartmentalization in the biosynthesis of CPC in *A. chrysogenum* [2,14] particularly in the transport of penicillin (IPN and PenN) across the peroxisomal membrane. The confocal microscopy experiments reported here clearly indicate that the microbodies reported in our previous article [14] are authentic peroxisomes since the p43EGFP-SKL peroxisome-targeted co-localizes with the DsRed fluorescent CefP hybrid protein. The role of CefP protein is probably to transport IPN from the cytosol to the perosixomal matrix were it is epimerized to form PenN. Finally, the CefM protein [14] secretes the epimerization product (PenN) from the peroxisomal lumen to the cytosol were it is converted into CPC by the expandase-hidroxylase [9] and DAC acetyltransferase [10] activities. We may conclude that peroxisomes play an indispensable role in the biosynthetic pathway of CPC in *A. chrysogenum* since in them seem to contain the enzymes that catalyse the epimerization step as demonstrated by the CefM [14] and CefP carriers characterization. The usefulness of this compartmentalization in the CPC biosynthetic process in *A. chrysogenum*, like in *P. chrysogenum* for penicillin biosynthesis, is to allow division of precursors and enzymes and to aid in the regulation of the reactions involved. Those advances in the compartmentalization of β-lactam biosynthetic enzymes in fungi, may serve as model for the localization of other secondary metabolite biosynthetic enzymes in microbodies of plants and other fungi.

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Table 1 CPC synthesis in cell-free extracts of the *cefP* mutant and the parental strain.

Strain	Genotype	CPC formed ng CPC/mg of cell dry weight
A. chrysogenum C10	Parental high cephalosporin producer	8.12±0.8
A. chrysogenum TDP-115	*ΔcefP*$^{-}$	9.42±0.7

The strains were grown in DP medium and cells were collected at 120 hours. Data are the average of three determinations. The conversion of IPN to CPC in vitro was quantified as described previously (14).

Table 2 Strains obtained with the complementation of the TDP-115 mutant.

Strain	Plasmid integrated	Complemented mutation
A. chrysogenum TDP-115	None	None
A. chrysogenum TCP-12	pCP	*ΔcefP*
A. chrysogenum TCR-45	pCR	*ΔcefR*
A. chrysogenum TCPR-27	pCRP	*ΔcefP* and *ΔcefR*

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LEGENDS TO THE FIGURES

Figure 1 β-lactam production in liquid cultures of the *cefP*-disrupted mutants and the parental strain. Cephalosporins (panels A, B and C) and extracellular penicillin (mixture of IPN and PenN) production (D and E) of *A. chrysogenum* C10 and the disrupted mutants (TDP-115, TDP-206). The error bars indicate the standard deviations of data from three independent cultures. In panel **A** analysis of the total cephalosporins production (CPC and DAC) in the culture broths was made by bioassay using *E. coli* ESS2231 as test strain. Panels **B** and **C** show HPLC analysis of the CPC (B) and DAC (C). Panel **D** shows extracellular penicillin (IPN+PenN) production in the culture broths. **(E)** HPLC analysis of the extracellular IPN (black bars) and PenN (white bars).

Figure 2 HPLC analysis of the intracellular DAC (A) and extracellular CPC (B) production in the TDP-115 and TDP-206 disrupted mutants and *A. chrysogenum* C10.
The error bars indicate the standard deviations of data from three independent cultures. Note the lack of CPC production in the two disrupted transformants.

Figure 3 Southern blot and RT-PCR analysis of *cefP* complementation and cephalosporins production of the complemented strains.
(A) Plasmid pCP containing the *cefP* gene used in the complementation. Southern blot hybridization of *Sma*I digested genomic DNA from six trasformants (three transformants of each TDP strain), untransformed *A. chrysogenum* C10, and the disrupted mutants (TDP-115 and TDP-206), using as probe the 4.6 kb *Sma*I containing the *cefP* gene. Lanes 1, TCP-105; 2, TCP-191; 3, TCP-211; 4, TCP-8; 5, TCP-12; 6, TCP-81; Controls: Lanes 7, TDP-206; 8, TDP-115; 9, *A. chrysogenum* C10; Lane M, size markers (Lambda DNA / *Hind*III digested). The sizes of the hybridization bands are indicated on the right. **(B)** Cephalosporins production (CPC and DAC) by bioassay against *E. coli* ESS-2231 of untransformed *A. chrysogenum* C10, TDP-115, TDP-206 and mutants complemented with the *cefP* gene: TCP-105 (derived from TDP-206) and TCP-12 (from TDP-115) **(C and D)**. RT-PCR analysis of expression of the *cefP* (C) and *cefR* (D) in the lanes 1, *A. chrysogenum* C10; 2, TDP-115; 3, TCP-12 and 4, TCPR-27 strains.

Figure 4 Beta-lactam production in liquid cultures of the complemented mutants (TCP-12, TCR-45 and TCPR-27), the *cefP*-disrupted strain (TDP-115) and the control *A. chrysogenum* C10.
Bioassay analysis of the volumetric **(A)** and specific **(B)** cephalosporins production (CPC and DAC). Analysis by HPLC of the production of CPC **(C)**, DAC **(D)** and penicillins (IPN and PenN) **(E)**. **(F)** Extracellular IPN and PenN production. **(G)** Intracellular IPN and PenN production. The error bars indicate the standard deviations of data from three independent cultures.

Figure 5 Subcellular localization of the CefP protein.

Hyphae of *A. chrysogenum* TPDsRed-32 strain obtained from cultures grown for 72 h in DP medium were observed by phase-contrast microscopy and confocal laser scanning fluorescence microscopy. Comparison of merged images (phase-contrast and fluorescence images) of eGFP-SKL **(A)**, CefP-DsRed **(B)** and both fluorescent proteins **(C)**. eGFP and DsRed fluorescence co-localized (yellow color), indicating that both fusion proteins are present in peroxisomes.

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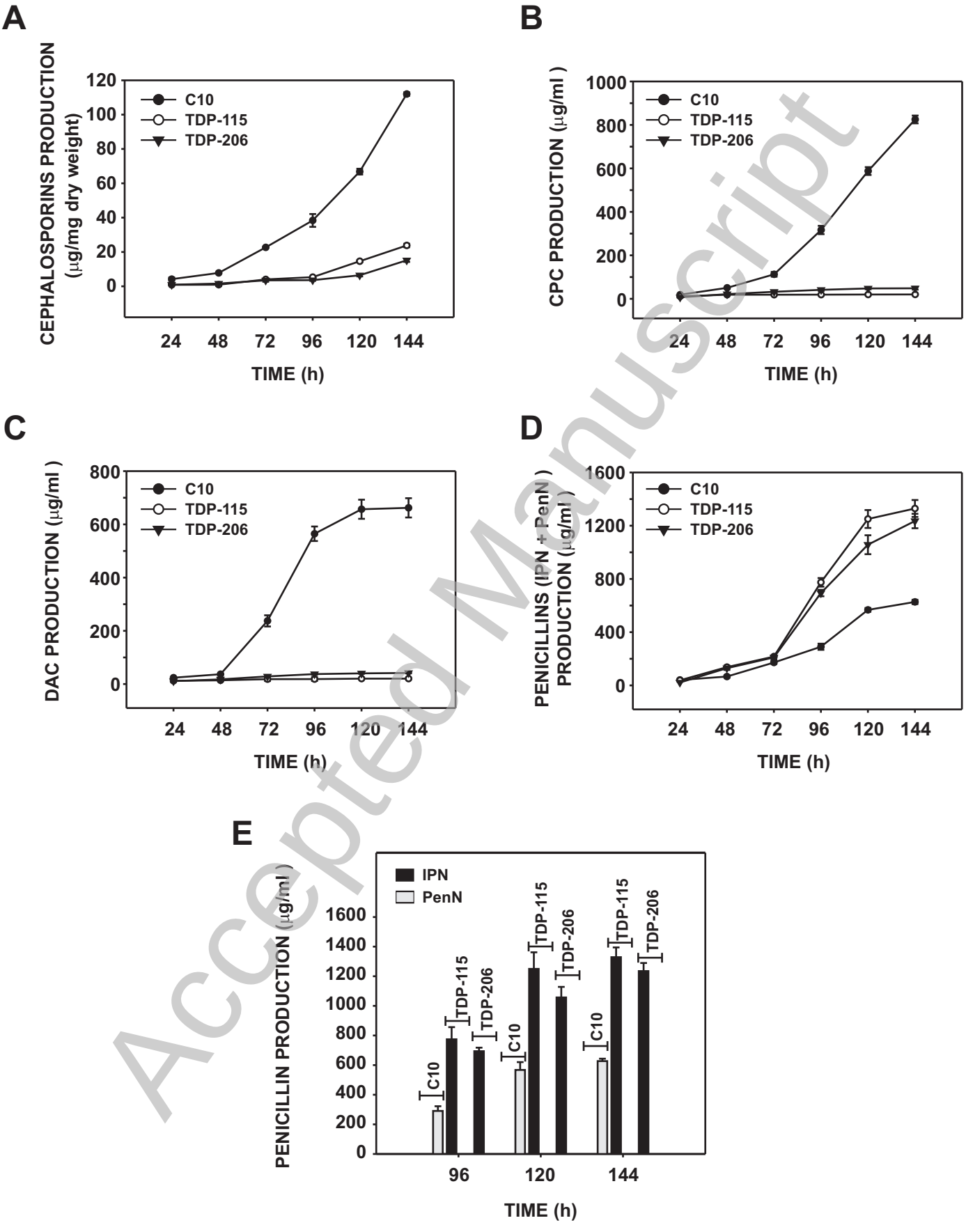

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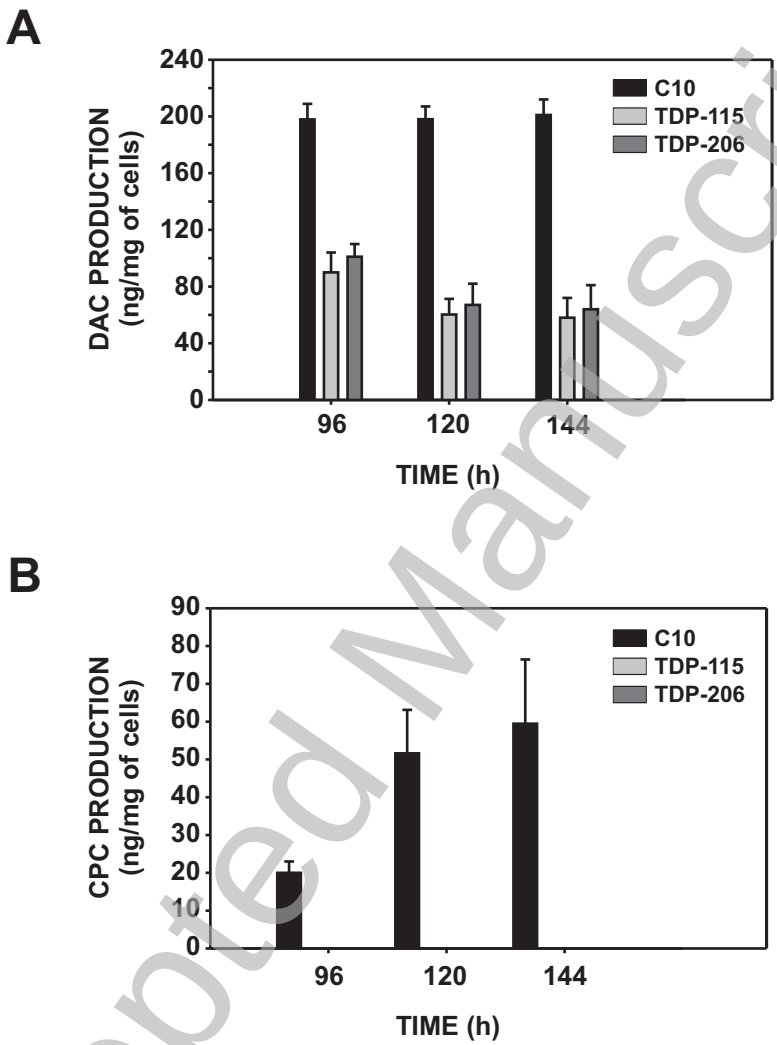
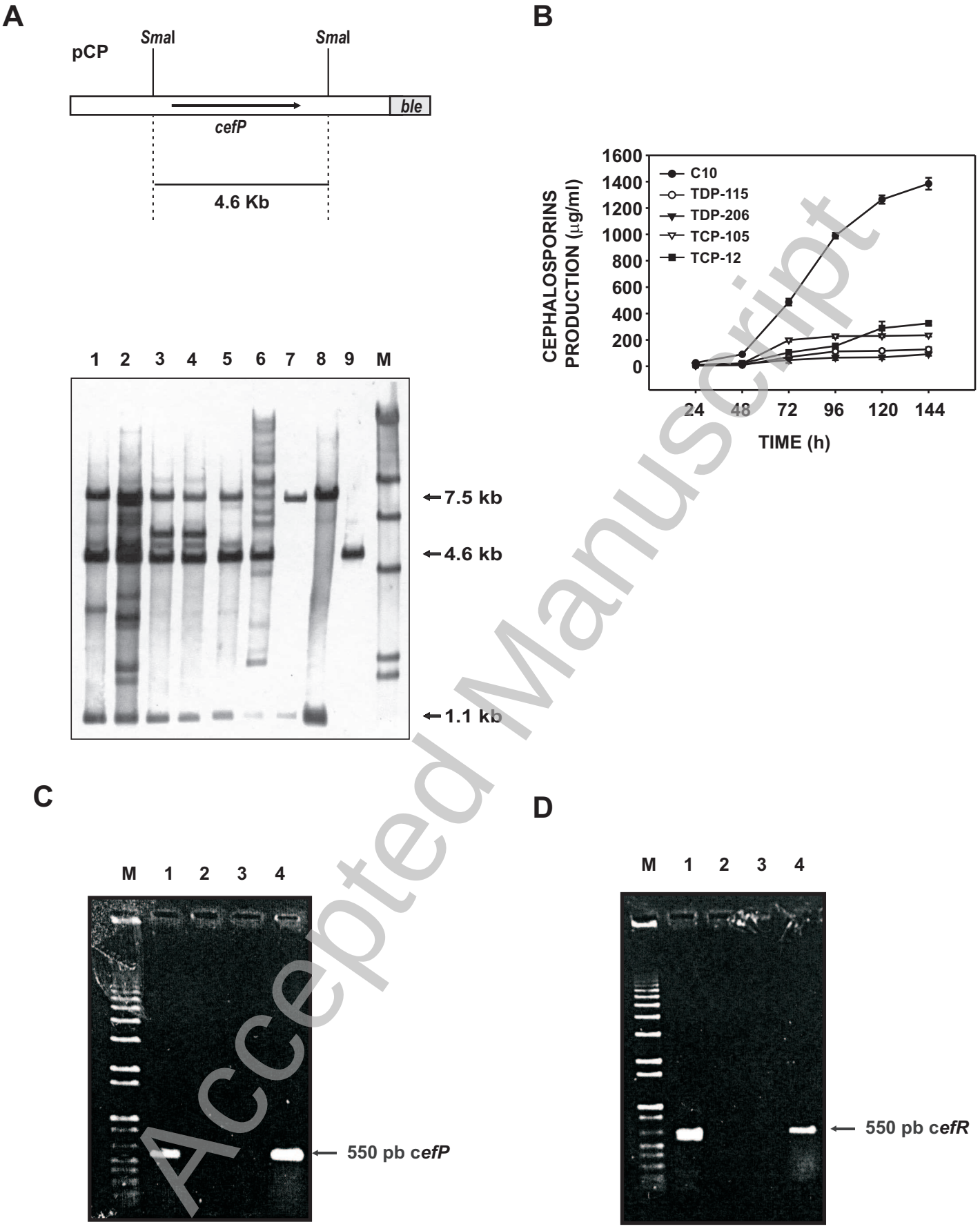


Ullán et al. - Figure 2



Ullán et al. - Figure 3

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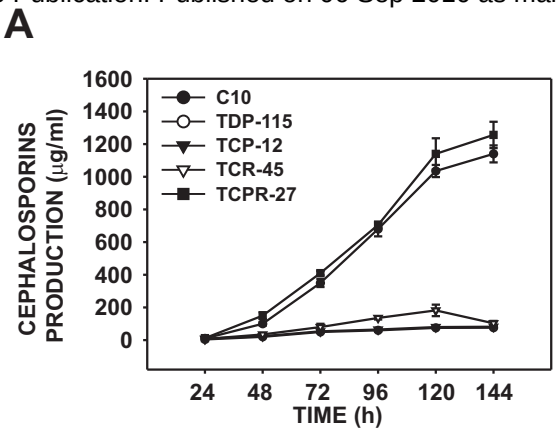

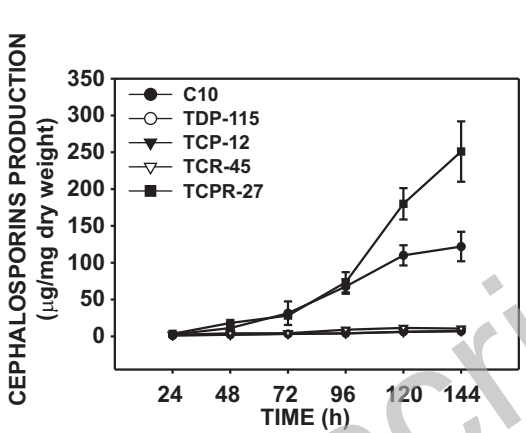

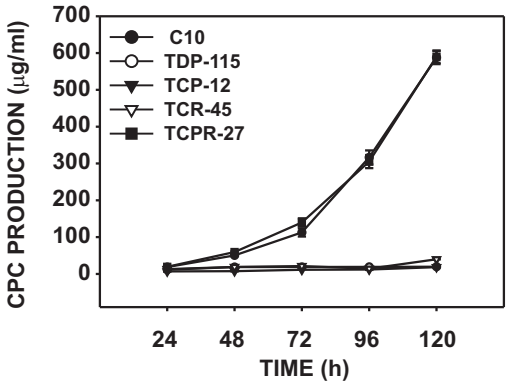

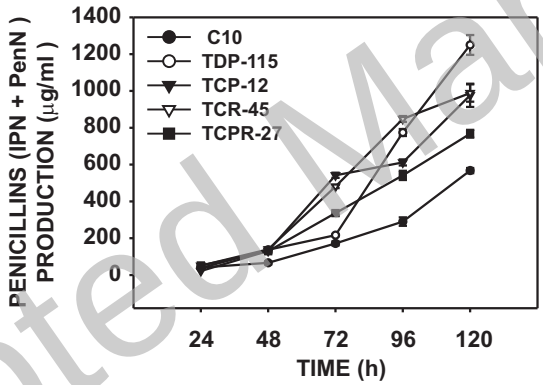

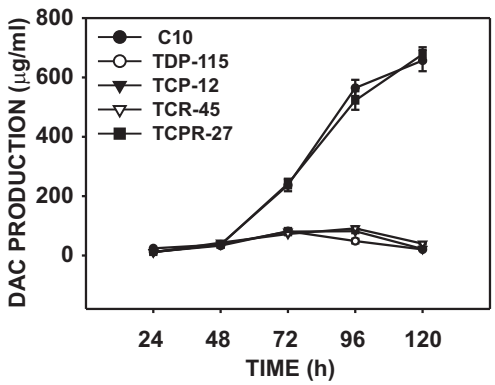

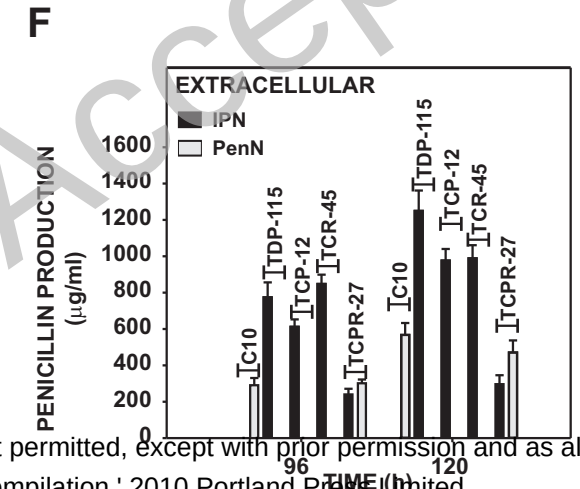

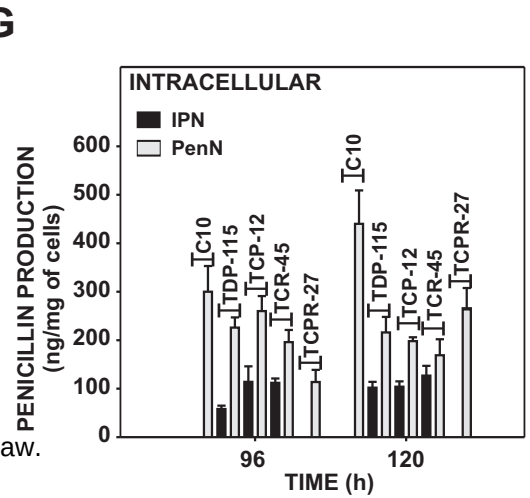

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