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ORIGINAL PAPER

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The *cefT* gene of *Acremonium chrysogenum* C10 encodes a putative multidrug efflux pump protein that significantly increases cephalosporin C production

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Abstract Transcriptional analysis of the region downstream of the *pcbAB* gene (which encodes the α-aminoadipyl-cysteinyl-valine synthetase involved in cephalosporin synthesis) of *Acremonium chrysogenum* revealed the presence of two different transcripts corresponding to two new ORFs. ORF3 encodes a putative D-hydroxyacid dehydrogenase and *cefT* (for *t*ransmembrane protein) encodes a multidrug efflux pump belonging to the Major Faciltator Superfamily (MFS) of membrane proteins. The CefT protein has 12 transmembrane segments (TMS) and contains motifs A, B, C, D2 and G characteristic of the Drug:H^+ antiporter 12-TMS group of the major facilitator superfamily. The CefT protein confers resistance to some toxic organic acids, including isovaleric acid and phenylacetic acid. Targeted inactivation of ORF3 and *cefT* by gene replacement showed that they are not essential for cephalosporin biosynthesis. However, amplification of the *cefT* gene results in increments of up to 100% in cephalosporin production in the *A. chrysogenum* C10 strain. Amplification of a truncated form of the *cefT* insert did not lead to cephalosporin overproduction. It seems that the CefT protein is involved in cephalosporin export from *A. chrysogenum* or in transmembrane signal transduction, and that there are redundant systems involved in cephalosporin export.

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Introduction

Cephalosporin C is a β-lactam antibiotic produced industrially by the filamentous fungus *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*). Cephalosporin biosynthesis begins with the condensation of the three precursor amino acids L-α-aminoadipic acid, L-cysteine and D-valine to form the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV; Aharonowitz et al. 1992; Martín 2000). This reaction is catalyzed by the ACV synthetase (Baldwin et al. 1990) which is encoded by the *pcbAB* gene (Gutiérrez et al. 1991b). The tripeptide is cyclized to form isopenicillin N (IPN) by the IPN synthase encoded by the *pcbC* gene (Samsom et al. 1985). The IPN is then isomerized to the D-configuration, giving rise to penicillin N, which is later converted to deacetoxycephalosporin and deacetylcephalosporin C by a bifunctional enzyme named deacetoxycephalosporin C synthase-hydroxylase (Scheidegger et al. 1984; Dotzlaf and Yeh 1987; Samsom et al. 1987). The last step in the cephalosporin biosynthesis pathway involves the conversion of DAC to cephalosporin C by the DAC acetyltransferase that is encoded by the *cefG* gene (Gutiérrez et al. 1992; Matsuyama et al. 1992; Velasco et al. 1999).

Nothing is known about the mechanism of cephalosporin export in *Acremonium chrysogenum*. The lack of information on the export of β-lactam antibiotics from fungal cells is amazing, considering the industrial interest of these compounds. In the cephamycin (7-methoxycephalosporin)-producing actinomycetes *Streptomyces clavuligerus* and *Amycolatopsis lactamdurans*, export of the antibiotic is believed to be mediated by a membrane protein encoded by the *cmcT* gene located in the cephamycin cluster (Coque et al. 1993; Pérez-Llarena et al. 1997). In *Aspergillus nidulans* an

ABC transporter encoded by the *atrD* gene has recently been reported to be involved in penicillin secretion (Andrade et al. 2000).

A growing number of multidrug efflux systems – secondary transporters driven by the proton motive force of the transmembrane electrochemical gradient – have been identified (Littlejohn et al. 1992; Lomovskaya and Lewis 1992; Grinus and Goldberg 1994). These proton-dependent multidrug transporters share an ability to export a wide variety of structurally unrelated substances out of the cell. These transporters are structurally diverse and belong to one of the following protein families: (1) the Major Facilitator Superfamily (MFS), (2) the Small Multidrug Resistance (SMR) family, and (3) the Resistant Nodulation (RND) family (Paulsen et al. 1996; Pao et al. 1998).

Antibiotic resistance in some antibiotic-producing bacteria is mediated by efflux mechanisms that extrude the antibiotic from the cell (Cundliffe 1989). In human cells, semisynthetic cephalosporins with a quaternary nitrogen have been found to serve as substrates for an organic cation/carnithine transporter (Ganapathy et al. 2000); this transporter might be related to proteins involved in the secretion of the natural cephalosporin C.

Most of the genes involved in antibiotic biosynthesis in many microorganisms are organized in clusters (Martín and Liras 1989; Martín 1992; Keller and Hohn 1997). In *A. chrysogenum* the genes *pcbAB* and *pcbC* are located on chromosome VII in the so-called cluster of the early cephalosporin biosynthetic genes (Fig. 1; Gutiérrez et al. 1991b), while the *cefEF* and *cefG* genes are located on chromosome I (Gutiérrez et al. 1992, 1999). In order to search for proteins involved in cephalosporin export or regulation, we have characterized the region located downstream of the *pcbAB* gene. In this communication, we report for the first time the characterization of a gene located in the cluster of early cephalosporin genes which encodes an MFS protein in *A. chrysogenum*, and investigate its role in cephalosporin C production.

Materials and methods

Microorganisms and culture media

Acremonium chrysogenum C10 (ATCC 48272), a high-cephalosporin-producing strain released by PanLabs (Demain 1983; Ramos et al. 1986), was used in this work. *A. chrysogenum* and its transformants were grown in LPE medium for 7 days at 28°C for sporulation. Spores collected from three plates of LPE medium were suspended in 100 ml of seed medium (Shen et al. 1986) in 500-ml shake-flasks and incubated at 25°C for 48 h in an orbital incubator at 250 rpm. Aliquots (5-ml) of the seed medium were used to inoculate 100-ml portions of MDFA medium (Shen et al. 1986) in 500-ml shake-flasks, which were incubated at 25°C for 6 days (in triplicate). Samples were taken every 24 h and production of β-lactam antibiotics was monitored by bioassay against *E. coli* ESS2231.

Plasmids and vectors for gene disruption and complementation

pBluescript KS(+) (Stratagene) was used for subcloning experiments. Plasmid pAN7-1 (Punt et al. 1987) containing the *hph* (for hygromycin resistance) gene was used for construction of pD3 and for co-transformation experiments.

For *cefT* gene replacement, plasmid pD3 was constructed. A 5.2-kb *Not*I-*Bam*HI DNA fragment containing the 5′ region of ORF7 and a 3.6-kb *Bam*HI DNA fragment containing the 5′ part of ORF3 of the early cephalosporin cluster (Fig. 1) were inserted into pBluescript KS(+). In this construct a 2.65-kb DNA fragment containing ORF3 and part of ORF7 was replaced by the *ble* gene (for bleomycin or phleomycin resistance) under the control of the *pcbC* gene promoter of *A. chrysogenum* isolated from pAC43 (S. Gutiérrez and J. F. Martín, unpublished results). A second selection marker (the *hph* gene isolated from pAN7-1) was inserted into the *Not*I site of the polylinker of this construct to form pD3.

For complementation studies the 1.8-kb *Nco*I-*Eco*RV fragment containing the intact ORF3 and the 2.4-kb *Eco*RI fragment containing the intact ORF7 were separately cloned into pBluescript

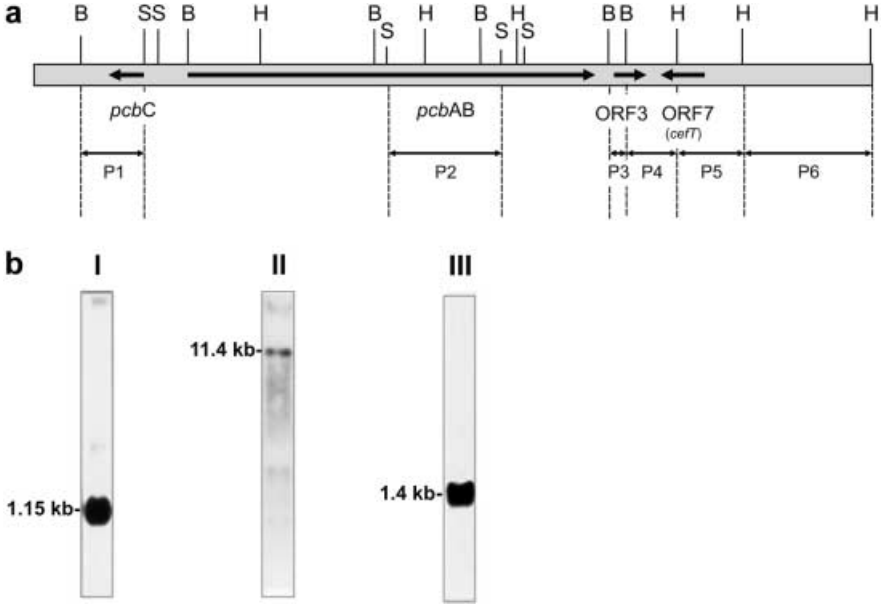


Fig. 1. **A** Region of *Acremonium chrysogenum* containing the cluster of early cephalosporin biosynthesis genes *pcbAB*, *pcbC*, ORF3 and ORF7 (*solid arrows*). H, *Hin*dIII; S, *Sal*I; B, *Bam*HI. The fragments used as probes P1–P6 in the transcript analysis are indicated by the *thin arrowed lines*. **B** Northern analysis of total RNA extracted from *A. chrysogenum* C10 with probes P1 (panel I), P2 (panel II) and P3 or P4 (panel III). The hybridization signal with probe P5, corresponding to ORF7 (*cefT*), was barely detectable

KS(+) to form the plasmids pORF3 and pORF7, respectively. Similarly, pORF7+3 was constructed by inserting into pBluescript KS(+) a 4.2-kb DNA fragment containing the entire ORF3 and ORF7.

A. chrysogenum transformation

Transformation of *A. chrysogenum* protoplasts was performed as described previously (Gutiérrez et al. 1991a). Transformants were selected in tryptic soy agar (TSA, Difco) supplemented with sucrose (10.3%) and phleomycin (10 μg/ml).

Isolation of *A. chrysogenum* genomic DNA

A. chrysogenum spores from a single plate were inoculated into 100 ml of MDFA medium (Shen et al. 1986). After incubation in an orbital shaker at 200 rpm for 48 h at 25°C the mycelium was collected by filtration through a Nytal filter (30 μm pore diameter), dried and lyophilized. Lyophilized mycelium (500 mg) was combined with 1 ml of grinding solution (0.18 M TRIS-HCl pH 8.2, 10 mM EDTA, 1% SDS) and 1 ml of phenol:chloroform:isoamyl alcohol (25:24:1 by volume) (Phenol-CIA), mixed well and incubated at 50°C for 30 min. Phenol-CIA treatment was repeated until the interface was free of proteins. The DNA was precipitated with ethanol and resuspended in 100 μl of TE buffer (Sambrook et al. 1989).

Southern blotting and hybridizations

Aliquots (3 μg) of genomic DNA of *A. chrysogenum* C10 or the transformants derived from it were digested with the appropriate enzymes and fractionated in an 0.8% agarose gel. The gel was blotted onto Hybond-N membrane as described by Sambrook et al. (1989). Digoxigenin labeling, hybridization and detection were done with the Genius kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridizations were performed at 68°C and the blots were washed twice with 2×SSC-0.1% SDS for 5 min at room temperature and twice with 0.1×SSC-0.1% SDS for 15 min at 68°C. When quantification was required probes were labeled by nick-translation with [α-^{32}P]dCTP and hybridizations were performed by standard methods (Sambrook et al. 1989). The intensity of the hybridization signals was determined by using a phosphorimager scanner (Instant Imager, Packard).

DNA sequencing

A nested set of deletion fragments from plasmids pS1a/b (see Results) were generated using the Erase-a-Base procedure (Promega) by digestion with Exonuclease III (Henikoff 1984). The fragments were sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1977), using an Autoread sequencing kit (Amersham-Pharmacia Biotech).

RNA isolation and intron analysis

Total RNA was isolated from *A. chrysogenum* C10 grown in MDFA medium for 72 h with the RNeasy kit (Qiagen). To confirm the presence of a putative intron, the DNA regions around the expected splicing site were amplified by RT-PCR using mRNA as a template and the primers 5′-TCAACAACATCGACACCG-3′ and 5′-AGTAGAGCCAGCGCC-3′, and sequenced to confirm the position of the splicing site.

Results

Transcriptional analysis of the region downstream of *pcbAB*

The presence of transcribed genes located downstream of the *pcbAB* (encoding the ACV synthetase) was studied by transcriptional analysis of this DNA region in *A. chrysogenum* C10. Six probes, P1–P6 (Fig. 1), were used to analyze this region (Table 1).

The P1 probe hybridized with the *pcbC* transcript (1.15 kb). The large 11.4-kb transcript corresponding to the *pcbAB* gene hybridized with the P2 probe. In addition, a new transcript of 1.4 kb was found that gave hybridized with probes P3 and P4.

This novel 1.4-kb transcript showed a high hybridization intensity, equivalent to that of *pcbC* (which is highly expressed during cephalosporin biosynthesis; Gutiérrez et al. 1991b), indicating that this new gene is transcribed efficiently in strain C10. This transcript corresponds to ORF3 (see below). An additional transcript corresponding to ORF7 was later shown to hybridize with probe P5, but its level of expression was barely detectable and much lower than that of ORF3, suggesting that ORF7 is very weakly expressed. No transcripts were detected with probe P6, suggesting that this fragment corresponds to an intergenic region.

Identification of the two ORFs downstream of the *pcbAB* gene

Two *Hin*dIII fragments (4.5 and 1.85 kb long) of the region located downstream of the *pcbAB* gene (Fig. 1) were cloned into the pBluescript KS (+) in both orientations, giving rise to plasmids pS1a and b and pS2a or b. The inserts of pS1 and pS2 were completely sequenced on both strands. The nucleotide sequence was deposited in the EMBL database under the Accession nos. AJ487683 (cefT) and AJ487684 (ORF3).

Analysis of the nucleotide sequence of the 6.35-kb DNA insert revealed the presence of two convergent ORFs, initially named ORF3 and ORF7 (Fig. 1). ORF3 (1047 nt) has no introns, and encodes a protein of 348 amino acids with a deduced molecular mass of 38 kDa. The amino acid sequence of the protein encoded by ORF3 showed strong similarity with 2-D-hydroxyacid dehydrogenases. The ORF3 protein is 49.8% identical to the 2-D-hydroxyacid dehydrogenase of *Zymomonas mobilis* (Yomano et al. 1993), and shows 33.1% identity to D-lactate dehydrogenase of *Lactobacillus plantarum* (Hummel et al. 1985), 32.2% identity to the 2-D-hydroxyisocaproate dehydrogenase

Table 1. Probes used to detect transcripts in the region downstream of the *pcbAB* gene

Probe	Size of hybridizing fragment	Transcript length	Gene
P1	1.8-kb *Sal*I-*Bam*HI	1.15 kb	*pcbC*
P2	3.2-kb *Sal*I	11.4 kb	*pcbAB*
P3	0.5-kb *Bam*HI	1.4 kb	ORF3
P4	1.3-kb *Hin*dIII-*Bam*HI	1.4 kb	ORF3
P5	1.8-kb *Hin*dIII	2.5 kb[a]	ORF7
P6	3.5-kb *Hin*dIII	None	None

[a]Very weak signal

of *L. casei* (Hummel et al. 1985) and 37.3% identity to the D-specific α-ketoacid dehydrogenase of *Enterococcus faecium* involved in vancomycin resistance (Arthur et al. 1991). Comparative analysis showed strong conservation around the NAD-binding pocket of the dehydrogenases, in addition to six other motifs conserved throughout the protein.

ORF7 is 1745 nt long and is interrupted by the presence of one intron in the 5′ region. The presence of the intron was confirmed by RT-PCR using the oligonucleotides P1 and P2 as primers (see Materials and methods). An amplification product of 657 bp was obtained, confirming the presence of a 60-bp intron in this region. The ORF7 encodes a protein of 561 amino acids with a deduced molecular mass of 61.3 kDa. The amino acid sequence of the ORF7p (Fig. 2) showed strong similarity throughout its entire length to multidrug-resistance proteins of *Gibberella pulicaris* (35.8% amino acid sequence identity), *Schizossacharomyces pombe* (31.2%), *Candida albicans* (25.7%) and *C. maltosa* (25.5%).

The ORF7-encoded protein is a member of the DHA12 facilitator superfamily

The protein encoded by ORF7 shows strong similarity to the members of a superfamily of multidrug efflux proteins that have the ability to export a variety of unrelated drugs from cells. The major facilitator superfamily (MFS) consists of symporters, antiporters and uniporters with 12 or 14 membrane-spanning segments (Paulsen et al. 1996; Pao et al. 1998). Computer analysis of the ORF7-encoded protein with the programs "TMHMM-prediction of transmembrane helix" (http://www.cbs.dtu.dk/services/TMHMM/) and "TopPred2-topology prediction of membrane proteins" (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) showed 12 transmembrane segments (TMS) (11 well supported and one doubtful according the second program used) (Fig. 2). A second feature of the MFS family is a motif (rxGRKxxI, where r indicates an arginine that is not strictly conserved, and x indicates any amino acid) conserved at equivalent positions within the cytoplasmic loop joining two transmembrane segments (Henderson and Maiden 1990; Griffith et al. 1994). In the ORF7p this motif is present as V^{147}YGRRIIY^{153} between TMS2 and TMS3 (Fig. 2).

Family 3 of the MFS superfamily is the Drug:H^+ antiporter 12-spanner (DHA12) protein group (Pao et al. 1998), characterized by 12 TMS and the presence of motifs A, B, C, D_2 and G (Paulsen and Skurray 1993). Figure 2 shows that all these motifs are present in the ORF7p, although motif G shows some variations with respect to the consensus. These results, together with the overall sequence similarities and the presence of 12 TMS, indicate that the ORF7-encoded protein belongs to the DHA12 family.

Targeted inactivation of ORF3 and ORF7: isolation of transformant T43 with a complete deletion of ORF3

To determine whether ORF3p and ORF7p are involved in cephalosporin C biosynthesis, we inactivated both ORFs. Since targeted gene disruption is difficult to achieve in *A. chrysogenum* (Waltz and Kück 1993), we used the double marker technique (Mansour et al. 1988; Liu et al. 2001). For this purpose, a plasmid (pD3) was constructed, containing the phleomycin-resistance (*ble*) gene under the control of the *A. chrysogenum pcbC* promoter as transformation marker (Fig. 3A) and the *hph* (hygromycin resistance gene) under the control of the *gpd* gene promoter as a second selective marker (Punt et al. 1987). In plasmid pD3 the *ble* gene is flanked by two fragments of 5.2 and 3.6 kb homologous to the ORF3-ORF7 genomic region.

Plasmid pD3 was used to delete the ORF3 by double crossing-over and at the same time to inactivate the ORF7. After transformation, 100 colonies that were resistant to phleomycin were obtained. Three of these were phleomycin-resistant and hygromycin-sensitive, suggesting that a double recombination had taken place with the concomitant loss of the hygromycin resistance marker. These three clones were selected and tested for gene replacement.

If double crossing over takes place between the insert in the pD3 plasmid and the homologous region in the genome, the restriction/hybridization pattern should change. When genomic DNA is digested with *Bam*HI and hybridized with an internal fragment of ORF3 as probe, the parental strain, *A. chrysogenum*, should give rise to a 2.2-kb hybridizing band, while no hybridization signal should be obtained in the null (deletion) mutants. On the other hand, when the *ble* cassette is used as probe a 3.7-kb hybridization signal (corresponding to the genomic *pcbC* gene since the cassette contains the *pcb*C promoter) should be observed in the parental strain and signals at 3.7 and 5.1 kb (due to integration of the *ble* cassette) should be obtained in the disrupted strain. Hybridization analysis showed (Fig. 3B) that the parental *A. chrysogenum* C10 DNA hybridized with a 2.2-kb band when an 1.0 kb fragment internal to the ORF3 was used as probe (Fig. 3B, lane 4). A 3.7-kb hybridization signal was found in all the recombinants with pD3 when the *ble* resistance cassette was used as probe, confirming that the endogenous *pcbC* gene has not been disrupted (Fig. 3C, lane 4). In the T43 transformant (Fig. 3B, lane 2), no signal was observed when the ORF3 was used as probe and 3.7- and 5.1-kb bands were obtained when the *ble* cassette was used as probe (Fig. 3C, lane 2), showing the pattern expected of a null mutant. The ORF7 was truncated in this transformant, as shown by hybridization with an ORF7 probe (Fig. 3D).

Transformants T1 and T3 did not conform to the expected hybridization pattern for a deletion mutant and showed some hybridization with ORF3; they had

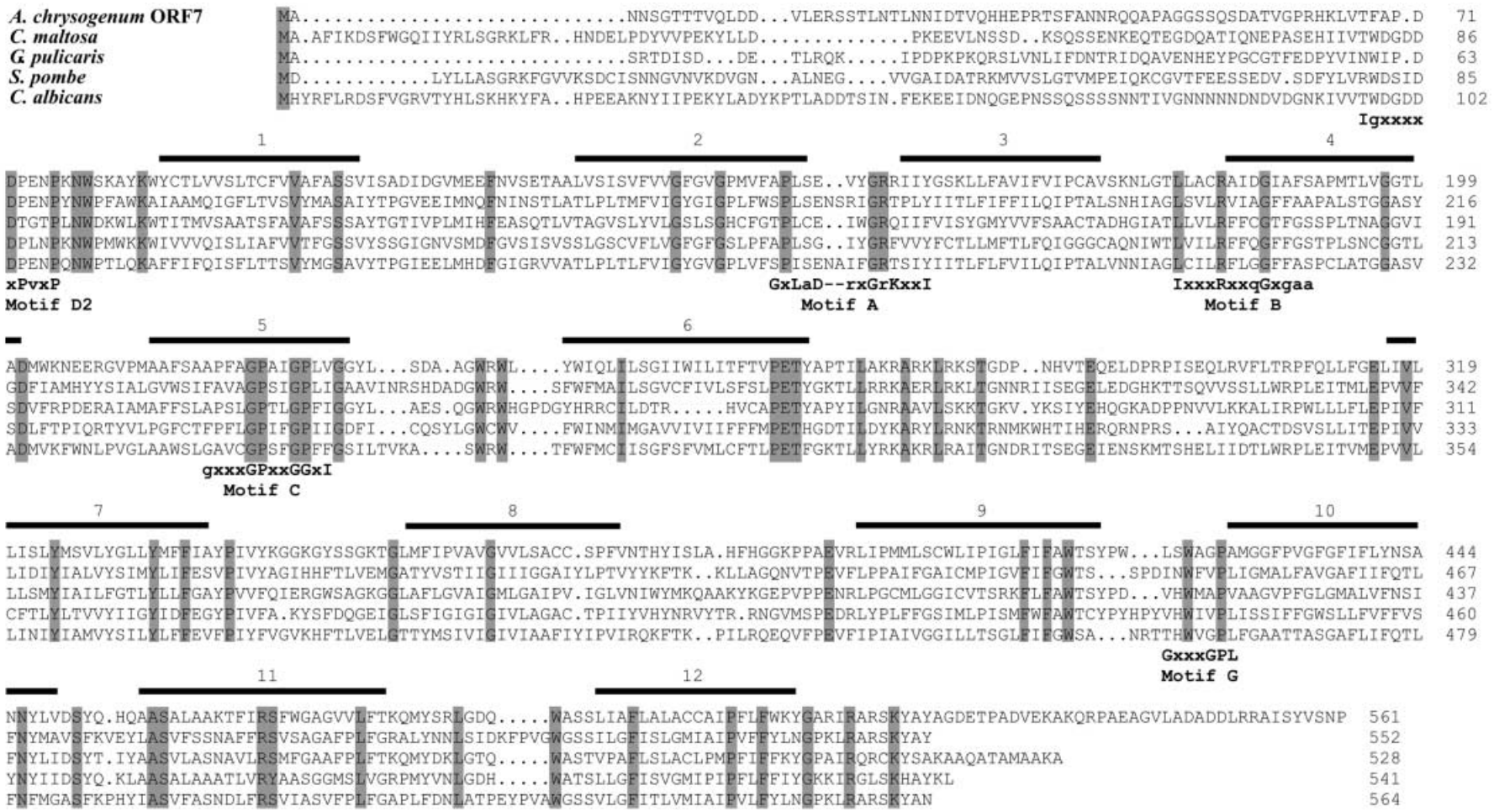


Fig. 2. Alignment of the amino acid sequence encoded by ORF7 with multiple drug resistance proteins of the MFS family from *Candida maltosa* (25.5% identity), *Gibberella pulicaris* (35.8%), *Schizossacharomyces pombe* (31.2%) and *C. albicans* (25.7%). Identical amino acids are *shaded*. The twelve transmembrane segments (TMS) are *overlined with solid bars*. Motifs A, B, C, D2 and G2, which are conserved in the members of the DHA12 family, are indicated in *bold below* the corresponding amino acid sequences

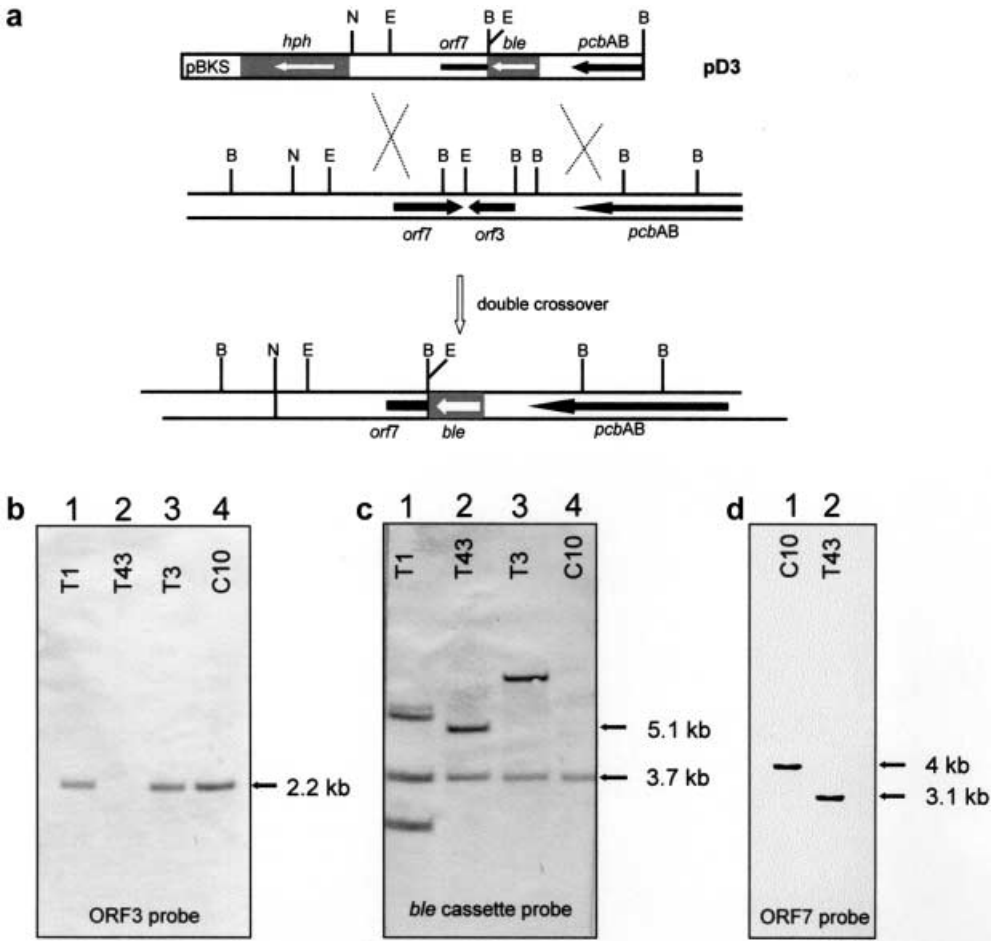


Fig. 3A–D. Deletion of ORF3 and disruption of ORF7. **A** Plasmid pD3 used for targeted inactivation of ORF7 and deletion of ORF3. The size of fragments expected after the double crossover is indicated. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sal*I. **B** Hybridization with the ORF3 probe of genomic DNAs from control *A. chrysogenum* C10 (lane 4) and three different transformants. Note that transformant T43 lacks the ORF3 hybridization signal. **C** Hybridization with the *ble* cassette as probe. The 3.7-kb band results from hybridization with the endogenous *pcbC* gene (used as internal standard) since the *ble* cassette contains the *pcbc* promoter to drive *ble* gene expression. **D** Hybridization with an ORF7 probe. Note the alteration of a 4.0-kb *Eco*RI band to a 3.1-kb *Eco*RI fragment corresponding to the truncated ORF7 in plasmid pD3. The DNA was digested with *Bam*HI in panels **B** and **C**; and with *Eco*RI in **D**

suffered unexpected recombinations and were not studied further.

Effect of ORF3 and ORF7 disruption and of complementation on cephalosporin C biosynthesis

Mutant T43 lacks active ORF7 and ORF3; therefore, in order to study the role of each of these ORFs we constructed strains with all the possible combinations of ORF3 and ORF7, i.e., mutant strains in which (1) ORF3 is inactive; (2) ORF7 is inactive; or (3) ORF3 and ORF7 functions are both restored.

The plasmids pORF7 (bearing ORF7), pORF3 (bearing ORF3) and pORF7+3 (bearing both ORFs) (Fig. 4) were prepared and introduced into mutant T43 (defective in ORF3 and ORF7) using the pAN7-1 vector (Punt et al. 1987) for co-transformation experiments. Ten transformants obtained in transformations with each plasmid (pORF7, pORF3 or pORF7+3) were selected at random, and a Southern analysis was performed to determine which of the transformants contained the plasmid inserts in the intact form. Three types of strains were generated in this experiment (Table 2): (1) T43-ORF7, in which ORF7 has been complemented but ORF3 is defective; (2) T43-ORF3, in which ORF3 has been complemented but ORF7 remains mutated; and (3) T43-ORF7+3 in which the mutant ORFs have both been complemented.

In order to study the effect of the ORF3 and/or ORF7 protein on cephalosporin C biosynthesis, *A. chrysogenum* C10, and the mutant strains T43, T43-ORF3, T43-ORF7 and T43-ORF7+3 were grown in MDFA medium. The cultures were incubated in parallel in triplicate flasks and the same fermentation was repeated three times. The results showed (Fig. 5A) that the growth rate is similar in all the strains in the absence of selective antibiotics, indicating that inactivation of ORF3 and ORF7 does not affect the growth of *A. chrysogenum*. However, cephalosporin C biosynthesis (Fig. 5B) was clearly enhanced in transformants T43-ORF7 and T43-ORF7+3. After incubation for 72 h, these transformants showed an increment of up to 40% in cephalosporin C production, as compared to *A. chrysogenum* C10, that was maintained throughout the fermentation. Therefore, the gene encoded by ORF7 has been named *cefT* for a transmembrane (T) protein that appears to be involved in cephalosporin secretion or regulation.

It is interesting to note that strain T43, which lacks both ORF3 and *cefT*, still produces cephalosporin

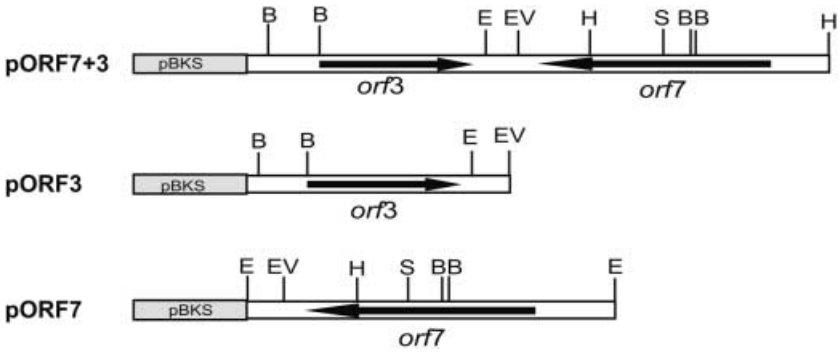


Fig. 4. Constructs used to complement the T43 mutant with ORF3, ORF7 or ORF7 + ORF3. In all cases the double transformants were identified by selecting for resistance to hygromycin

Table 2. Strains generated by complementation of the double disruption mutant T43

Strain	Plasmid integrated	ORF complemented	Remaining mutation
T43	None	None	*orf3* and *orf7*
T43-ORF7	pORF7	ORF7	*orf3*
T43-ORF3	pORF3	ORF3	*orf7*
T43-ORF7 + 3	pORF7 + 3	ORF7 and ORF3	None

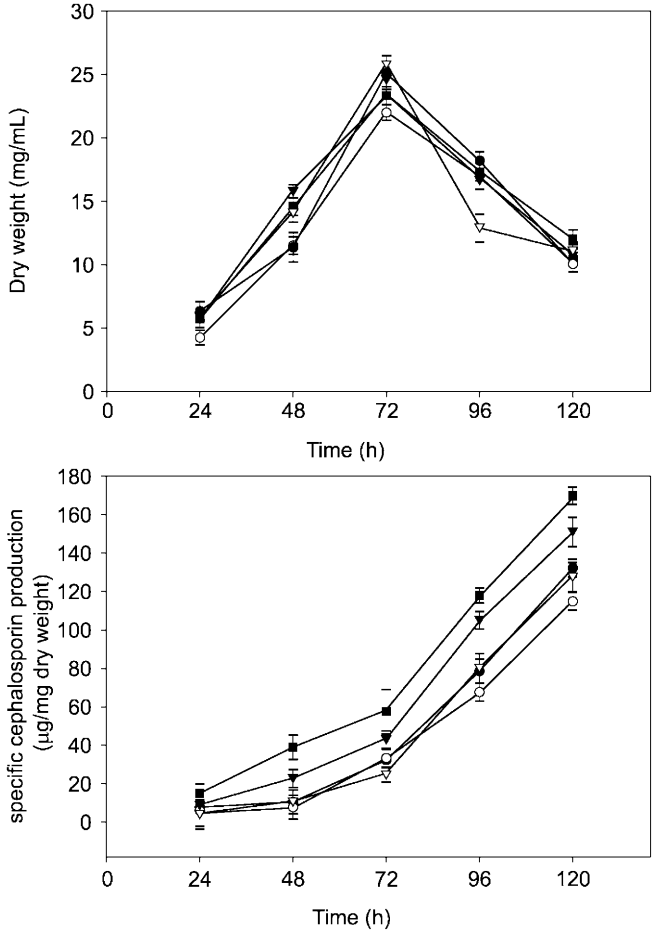


Fig. 5A, B. Growth kinetics (**A**) and specific cephalosporin production (**B**) of control *A. chrysogenum* C10 and several T43 transformants. Strain T43 contains the disrupted ORF7 and lacks ORF3. *Filled circles*, C10; *open circles*, T43; *open triangles*, T43-ORF3; *filled squares*, T43-ORF7; *filled triangles*, T43-ORF7 + 3

(at slightly reduced levels compared to the parental strain C10), indicating that if CefT is involved in cephalosporin secretion, redundant systems for the secretion of this antibiotic must be present in *A. chrysogenum*.

Characterization of the multidrug-resistant phenotype conferred by ORF7

Many of membrane DHA12 proteins belonging to the MFS family have been shown to play an important role in bacteria and eukaryotic cells by conferring resistance to toxic compounds (Paulsen et al. 1996).

In order to search for a phenotype conferred by the ORF7 protein, we tested the toxicity of several compounds in *A. chrysogenum* C10 and in the transformant strains T43, T43-ORF3, T43-ORF7 and T43-ORF7 + 3. The results showed (Table 3) that when the ORF7 was inactive, i.e. in strains T43 and T43-ORF3 isovaleric acid and phenylacetic acid were toxic for the fungus. However, when the ORF7 was present, i.e. in *A. chrysogenum* strains C10, T43-ORF7 and T43-ORF7 + 3, no toxic effect was detected when isovaleric acid or phenylacetic acid was added to MCFA or MDFA medium. The other tested compounds, including cephalosporin C, were inactive against *A. chrysogenum* and the disrupted mutants.

Amplification of *cefT* in *A. chrysogenum* C10

Since the T43-ORF7 mutant produced more cephalosporin C than *A. chrysogenum* C10 it was of interest to determine the effect of the copy number of this gene on cephalosporin production.

To study the effect of amplification of the *cefT* gene, plasmid pORF7 was introduced into *A. chrysogenum* C10 by cotransformation with the help of pAN7-1 (conferring resistance to hygromycin). After transformation nine transformants were randomly selected to study the number of extra copies of the *cefT* gene integrated into the genome. For this purpose Southern blots of *Eco*RI-digested DNA from the selected transformants and the parental *A. chrysogenum* C10 were hybridized with the 2.2-kb *Eco*RI fragment of the *cefT* gene as

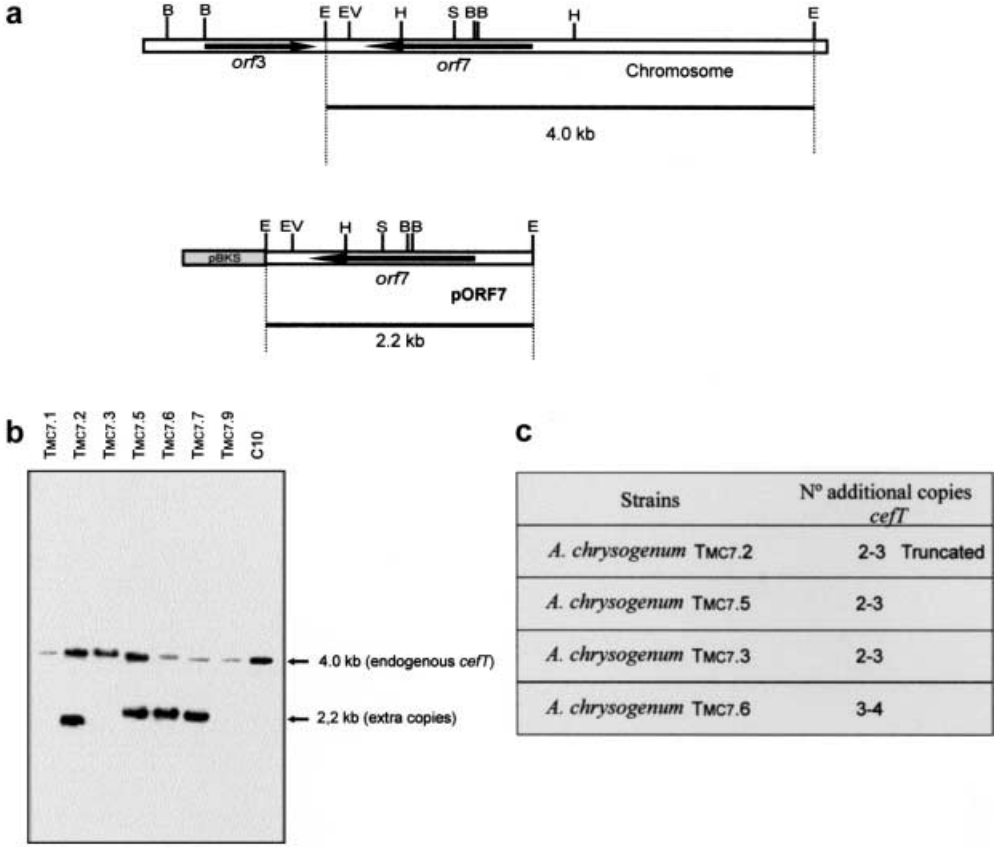
Table 3. Toxicity of phenylacetic acid (PAA) and isovaleric acid (IsoV) in different mutants

Medium[a]	Test strain (mutation)[b]				
	C10 (none)	T43 (orf7⁻ orf3⁻)	TORF7 (orf3⁻)	TORF3 (orf7⁻)	TORF7 + 3 (none)
MCFA	+	+	+	+	+
MCFA + PAA	+	–	+	–	+
MCFA + IsoV	+	–	+	–	+
MDFA	+	+	+	+	+
MDFA + PAA	+	–	+	–	+
MDFA + IsoV	+	–	+	–	+

[a]Phenylacetic acid (PAA) was added at a final concentration of 0.8% while isovaleric acid (IsoV) was added at 0.2% to both media
[b]The mutations remaining in each strain are indicated in *parentheses*. +, growth; –, no growth (-)

probe. Two hybridization bands should be obtained in the transformants: (1) a 4.0-kb band derived from the endogenous copy of *cefT*, and (2) a second band of 2.2 kb that corresponds to the *cefT* present in plasmid pORF7 when the insert is integrated in its intact form (Fig. 6A). Other hybridizing bands result from the integration of rearranged copies of the *cefT* insert in the pORF7 plasmid. The intensity of the 4.0-kb endogenous band was used as reference to quantify the number of extra copies of *cefT* integrated into the genome (Fig. 6B). The results showed that transformants TMC7.2, TMC7.5, TMC7.6 and TMC7.7 had extra copies of the *cefT* gene. Transformant TMC7.2 had 2 to 3 truncated copies of *cefT*, whereas transformants TMC7.5, TMC7.6 and TMC7.7 had, respectively, 2 to 3, 2 to 3 and 3 to 4 intact copies of *cefT* (Fig. 6B).

To analyze the effect of amplification of the *cefT* gene on cephalosporin production, fermentations were carried out in MDFA medium with *A. chrysogenum* C10 and the transformants TMC7.5, TMC7.6, TMC7.7 and TMC7.2 were performed (Fig. 7A and B). Transformants TMC7.5, TMC7.6 and TMC7.7 showed a clear increase of up to 100% in yields of cephalosporin C or total β-lactam (cephalosporins plus isopenicillin N and penicillin N intermediates) that correlated with the presence of 2–4 additional copies of the *cefT* gene (Fig. 6C). However transformant TMC7.2, which carries 2–3 copies of a truncated *cefT* did not show any



Strains	N° additional copies *cefT*
A. chrysogenum TMC7.2	2-3 Truncated
A. chrysogenum TMC7.5	2-3
A. chrysogenum TMC7.3	2-3
A. chrysogenum TMC7.6	3-4

Fig. 6A–C. Amplification of the ORF7 (*cefT*) gene in *A. chrysogenum* C10. **A** Diagram showing the endogenous 4.0-kb *Eco*RI fragment and the 2.2-kb *Eco*RI fragment containing ORF7 in plasmid pORF7 used for amplification of this gene. **B** Southern analysis using a *cefT* probe. **C** The number of additional copies of the *cefT* gene as estimated by comparison of the intensity of the 2.2-kb band with that of the endogenous 4.0-kb band. Note that transformant TMC7.2 contains several copies of a smaller (2.0 kb) insert (truncated ORF7)

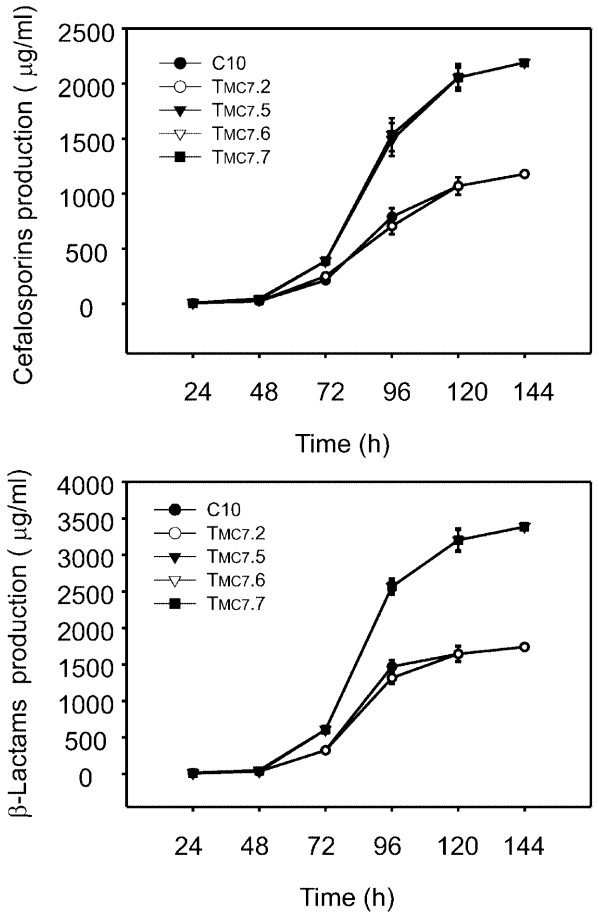


Fig. 7. Cephalosporin C (*upper panel*) and total β-lactam (*lower panel*) production in MDFA medium by different transformants. Note that TMC7.2 does not produce increased levels of cephalosporin C, whereas TMC7.5, TMC7.6 and TMC7.7 produce 80–100% higher levels than the untransformed *A. chrysogenum* C10

increase in cephalosporin production. In conclusion, a non-truncated *cefT* gene is required to obtain improved cephalosporin yields, thus eliminating the possibility that the increases in antibiotic yield are due to indirect effects of ectopic integrations.

Discussion

In order to advance our molecular knowledge of cephalosporin biosynthesis (Martín et al. 1999) we are searching for regulatory or export genes in the regions adjacent to the cephalosporin gene clusters, since regulatory and export genes are frequently linked to antibiotic biosynthesis genes (Martín and Liras 1989; Keller and Hohn 1997). Downstream from the *pcbAB* gene in the cluster of "early" cephalosporin genes we have found two ORFs; one of which (ORF3) encodes a putative 2-D-hydroxyacid dehydrogenase. Similar enzymes are known in bacteria and eukaryotes and appear to be involved in the catabolism of fatty acids or in the biosynthesis of D-amino acids. Targeted inactivation of this gene indicated that it is not essential for cephalosporin C biosynthesis. If ORF3 is involved in cephalosporin biosynthesis its function may be replaced by other redundant hydroxyacid dehydrogenases.

In this report we have also described a second ORF (ORF7) encoding a protein that belongs to the Major Facilitator Superfamily. This gene is located downstream of *pcbAB* and ORF3 but is oriented in the opposite direction; it has been named *cefT*. The deduced CefT protein shows a characteristic eight-residue motif (rxGRKxxI) located between the TMS2 and TMS3 on the cytoplasmic side of the membrane, as in other members of the MFS (Henderson and Maiden 1990; Griffith et al. 1994). The MFS is divided into 17 families (Pao et al. 1998). The CefT protein belongs to Family 3 of drug efflux proteins, the Drug:H^+ antiporter containing 12 membrane spanners (DHA12) family, since it has 12 putative membrane-spanning segments and also has the characteristic motifs (A, B, C, D_2, G) of the DHA12 family (Paulsen and Skurray 1993, 1996).

Functionally characterized members of the DHA12 family exhibit specificities for certain drugs, although the range of compounds transported is remarkably wide (Paulsen et al. 1996). The DHA12 family includes proteins that have been shown to be either multidrug resistance pumps or drug-specific efflux pumps. *cefT* seems to encode a multidrug resistance pump, because it confers resistance to at least two unrelated compounds (phenylacetic acid and isovaleric acid).

Targeted inactivation of *cefT* showed that it is not essential for cephalosporin C biosynthesis, suggesting that other cephalosporin export systems also occur in *A. chrysogenum*. When *cefT* was transformed into *A. chrysogenum* T43 or *A. chrysogenum* C10 extracellular cephalosporin accumulation is increased. The increase is more marked in strain C10, which also contains the functional endogenous copy of *cefT*.

Multiple copies of intact *cefT* increase cephalosporin production by up to a 100% in *A. chrysogenum* C10, but a truncated form of *cefT* did not. The stimulatory effect thus seems to require a functional CefT protein.

Some of the multidrug efflux systems of clinical relevance in drug-resistant microorganisms have been isolated. For instance, azole resistance in clinical *C. albicans* isolates is due to overexpression of the multidrug resistance *CaMDR1* gene (Sanglard et al. 1995) and resistance to fluoroquinolones in some *Staphylococcus* strains is partially due to overexpression of the multidrug resistance gene *norA* (Kaaz et al. 1993; Ng et al. 1994). In *A. chrysogenum* the CefT protein is involved in detoxification of isovaleric acid and phenylacetic acid, and may be also involved in the export of cephalosporin or cephalosporin intermediates. When the copy number of the *cefT* is increased the transporters could be active even in the absence of toxic compounds, i.e. the transporters are "empty" and could be used in the secretion of cephalosporin C or its biosynthetic intermediates through internal membrane systems or through the cytoplasmic membrane.

Recently it has been reported that the interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance (Lee et al. 2000). If a second transporter is involved in cephalosporin secretion (as suggested by the non-essential nature of *cefT*), amplification of *cefT* may result in increased cephalosporin export due to an additive effect.

Nothing is known about secretion of other β-lactam antibiotics in β-lactam-producing microorganisms, although genes that are functionally equivalent to *cefT* have been found in the cephamycin clusters of *Streptomyces clavuligerus* (Pérez-Llarena et al. 1997) and *Amycolatopsis lactamdurans* (Coque et al. 1993). In *P. chrysogenum* strains, high-penicillin-producing strains are less sensitive to feedback regulation by exogenously added penicillin than the wild-type strain, apparently because the penicillin is pumped out more efficiently (Revilla 1983). Recently, an ABC transporter has been reported to be involved in penicillin production in *A. nidulans* (Andrade et al. 2000).

Although a role for *cefT* in cephalosporin export is very likely, we can not yet exclude the possibility that the transmembrane CefT protein might be a sensor for extracellular signals triggering cephalosporin synthesis and, therefore, leads to increased extracellular accumulation when overexpressed.

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