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Expression of *cefD2* and the conversion of isopenicillin N into penicillin N by the two-component epimerase system are rate-limiting steps in cephalosporin biosynthesis

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Abstract The conversion of isopenicillin N into penicillin N in *Acremonium chrysogenum* is catalyzed by an epimerization system that involves an isopenicillin N-CoA synthethase and isopenicillin N-CoA epimerase, encoded by the genes *cefD1* and *cefD2*. Several transformants containing two to seven additional copies of both genes were obtained. Four of these transformants (TMCD26, TMCD53, TMCD242 and TMCD474) showed two-fold higher IPN epimerase activity than the untransformed *A. chrysogenum* C10, and produced 80 to 100% more cephalosporin C and deacetylcephalosporin C than the parental strain. A second class of transformants, including TMCD2, TMCD32 and TMCD39, in contrast, showed a drastic reduction in cephalosporin biosynthesis relative to the untransformed control. These transformants had no detectable IPN epimerase activity and did not produce cephalosporin C or deacetylcephalosporin C. They also expressed both endogenous and exogenous *cefD2* genes only after long periods (72–96 h) of incubation, as shown by Northern analysis, and were impaired in mycelial branching in liquid cultures. The negative effect of amplification of the *cefD1* - *cefD2* gene cluster in this second class of transformants is not correlated with high gene dosage, but appears to be due to exogenous DNA integration into a specific locus, which results in a pleiotropic effect on growth and *cefD2* expression.

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Introduction

Acremonium chrysogenum is used for the industrial production of cephalosporin. Cephalosporins are the major products in the β-lactam market, but the cephalosporin titers obtained are not very high when compared with the levels of penicillin synthesized by high-producer strains of *Penicillium chrysogenum* (Elander 2003).

The biosynthesis of cephalosporin C (CPC) in *A. chrysogenum* starts with the formation of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the non-ribosomal ACV synthetase (Gutiérrez et al. 1991b), followed by cyclization of ACV to isopenicillin N (IPN) (Aharanowitz et al. 1992). The IPN is later converted into penicillin N (PenN) by an epimerase system that has remained uncharacterized for many years (Lübbe et al. 1986; Ullán et al. 2002). After the epimerization step, PenN is converted into deacetoxycephalosporin C (DAOC) and deacetylcephalosporin C (DAC) by the action of a bi-functional enzyme with thiazolidine ring-expanding activity (expandase) and 3′-hydroxylase activity. Finally, DAC is converted into cephalosporin C by the DAC acetyltransferase (Martín and Demain 2002).

Recently, we reported that conversion of IPN into PenN in *A. chrysogenum* is catalyzed by a novel two-protein epimerization system (Ullán et al. 2002). The conversion of IPN into PenN involves the concerted action of an IPN-CoA synthetase encoded by the *cefD1* gene and an IPN-CoA epimerase encoded by the *cefD2* gene. This epimerization system is entirely different from the pyridoxal phosphate-dependent epimerase involved in the biosynthesis of bacterial β-lactam antibiotics (Jensen et al. 1983; Láiz et al. 1990; Martín et al. 2004),

but it is similar to epimerization systems involved in the degradation of rare fatty acids or toxic metabolites in other eukaryotic cells (Knihinicki et al. 1991, Shieh et al. 1993; Schmitz et al. 1995).

The cephalosporin biosynthetic genes in *A. chrysogenum* are organized in two clusters: *pcbAB*, *pcbC, cefD1* and *cefD2* are located in the so-called "early" cluster (encoding the enzymes that catalyze the early steps in the pathway), while *cefEF* and *cefG* genes are located in the so-called "late" cluster (Gutiérrez et al. 1992, 1999; Velasco et al. 1999). In *P. chrysogenum* a tandem amplification of the penicillin biosynthetic gene cluster has been found in high penicillin-producing strains (Barredo et al. 1989; Fierro et al. 1995; Newbert et al. 1997). However, in *Acremonium* only one copy of the cephalosporin biosynthetic genes is present even in improved strains such as *A. chrysogenum* C10 (Gutiérrez et al. 1992).

Results from various research groups have shown that distinct reactions can be rate-limiting in different β-lactam producers (Kennedy and Turner 1996; Gutiérrez et al. 1997). Therefore, we were interested in determining whether the new epimerization system might be a bottleneck in the cephalosporin biosynthesis pathway in *A. chrysogenum*.

In the work reported here, we have amplified the recently described *cefD1-cefD2* cluster in order to study if the IPN epimerase activity represents a rate-limiting step in cephalosporin biosynthesis in *A. chrysogenum*. Our results indicate that there is a clear correlation between an increase in isopenicillin N epimerase activity and cephalosporin overproduction. However, in some transformants, integration of extra copies of the *cefD1-cefD2* cluster leads to slow growth of unbranched mycelium, with very low expression of the *cefD2* gene and low levels of cephalosporin production.

Materials and methods

Microbial strains, media and growth conditions

A. chrysogenum C10 (ATCC 48272) is an improved cephalosporin C-producing strain released by PanLabs Laboratories (Ramos et al. 1986). *Escherichia coli* ESS2231 is a β-lactam-supersensitive strain, used for routine cephalosporin bioassays. *A. chrysogenum* strains were grown in LPE medium for 7 days at 28°C for sporulation. Spores and mycelium fragments collected from six plates of LPE medium were suspended in 100 ml of seed medium (Shen et al. 1986) in 500-ml shake-flasks for 48 h and agitated in an orbital incubator at 250 rpm. A 10-ml aliquot of seed culture was used to inoculate Shen's defined production (DP) medium (Shen et al. 1986). The cultures were incubated in triple-baffled flasks (500 ml; Bellco) containing 100 ml of medium at 25°C in a rotary shaker (at 250 rpm). Samples were taken every 24 h, and cephalosporin production was determined by bioassay using *E. coli* ESS2231 as the test strain on plates with penicillinase (from *Bacillus cereus* UL1) as described previously (Zanca and Martín 1983; Gutiérrez et al. 1997).

Plasmids

pCD1 + 2 contains a 5.8-kb *Bam* HI-*Eco*RV DNA fragment bearing the *cefD1* and *cefD2* genes (Ullán et al. 2002) inserted into the *Bam* HI/*Eco*RI site of the fungal vector pC43 (Gutiérrez et al. 1999). The *cefD1* and *cefD2* genes were expressed under the control of their own (bi-directional) promoter.

Transformation of *A. chrysogenum* protoplasts

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

Southern blotting and hybridization

Genomic DNA of *A. chrysogenum* was isolated as described previously (Gutiérrez et al. 1991a). Samples (3 μg) of genomic DNAs from *A. chrysogenum* C10 and its transformed derivatives were digested with restriction enzymes and fractionated by electrophoresis in 0.7% agarose gels. The gel was blotted onto Hybond-N membrane (Amersham-Biosciences) as described by Sambrook et al. (1989).

Hybridization probes were labeled with [α-^{32}P]dCTP by nick translation, and purified by filtration through Wizard mini-columns (Promega). Hybridizations were carried out as described by Sambrook et al. (1989). The intensity of hybridized bands was determined using a phosphorimager scanner (Instant Imager, Packard Instruments).

RNA extraction and Northern analysis

Total *A. chrysogenum* RNA was isolated with the RNeasy Kit (Qiagen). Total RNA was resolved by electrophoresis in agarose-formaldehyde gels and blotted onto Hybond-N membranes (Amersham Pharmacia Biotech) as described by Sambrook et al. (1989).

For Northern analysis the DIG Easy Hyb (Roche Diagnostics) was used. Hybridizations were performed according to the manufacturer's protocol. The hybridization signals were visualized by chemiluminescence and recorded on X-ray film with an exposure time of 10 min. The intensity of the hybridization bands was determined using a scanner (Hewlett-Packard) coupled to the GelPro analyzer 3.1 program (Media Cybernetics).

Determination of deacetylcephalosporin C and cephalosporin C by HPLC

The extracellular concentration of DAC and CPC produced by *A. chrysogenum* strains grown in DP medium was determined by HPLC in a Beckman System Gold chromatograph equipped with a μBondapack C18 column. The elution system used has been described previously (Gutiérrez et al. 1997).

Separation of isopenicillin N and penicillin N by HPLC

Isopenicillin N and penicillin N were identified by HPLC following the protocol described by Neuss et al. (1982). Isopenicillin N and penicillin N (500 μg/ml) were first derivatized with 2,3,4,6-tetra- *O* -acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (2 mg/ml in acetonitrile) for 2 h at pH 8.5 (adjusted with sodium bicarbonate). The derivatized compounds were then separated by HPLC in a Beckman System Gold high-pressure liquid chromatograph equipped with a μBondpack C18 column, using as the mobile phase a mixture of methanol-acetonitrile-acetic acid-water (36:7:2:55) at a flow rate of 1.2 ml/min.

IPN epimerase activity assay

The IPN epimerase assay was performed as described by Lübbe et al. (1986) and modified by Ullán et al. (2002). IPN epimerase activity was determined by measuring the IPN-dependent coupled conversion of IPN into CPC in vitro. Cell-free extracts were prepared from mycelium collected after 72 h of culture. One unit of activity corresponds to the formation of 1 ng of CPC per min in the coupled assay (Ullán et al. 2002). The enzymatic activity was expressed in units/mg of protein. The total protein concentration in cell extracts was measured by the Bradford assay (Bradford 1976).

Results

Amplification of the *cefD1-cefD2* gene cluster from *A. chrysogenum* C10

To determine if the epimerase activity is a limiting step in cephalosporin biosynthesis, the number of *cefD1* and *cefD2* genes encoding the IPN-CoA synthetase and IPN-CoA epimerase, respectively, was increased in a cephalosporin-producing strain. For this purpose plasmid pCD1 + 2 bearing the chromosomal region containing both the *cefD1* and *cefD2* genes from *A. chrysogenum* C10, under the control of the natural bi-directional promoter (Fig. 1), was introduced into *A. chrysogenum* C10, a strain that is known to express the other cephalosporin genes efficiently (Ramos et al. 1986), and transformants were selected by screening for resistance to phleomycin. The transformants were initially assayed by the agar plug method. Some transformants showed higher rates of cephalosporin production than *A. chrysogenum* C10, whereas many other transformants (about 80% of all clones) showed a drastic reduction in cephalosporin production. The number of intact copies of the insert containing the *cefD1* and *cefD2* genes present was determined in several randomly selected transformants by Southern hybridization analysis, using as probe a 1.0-kb

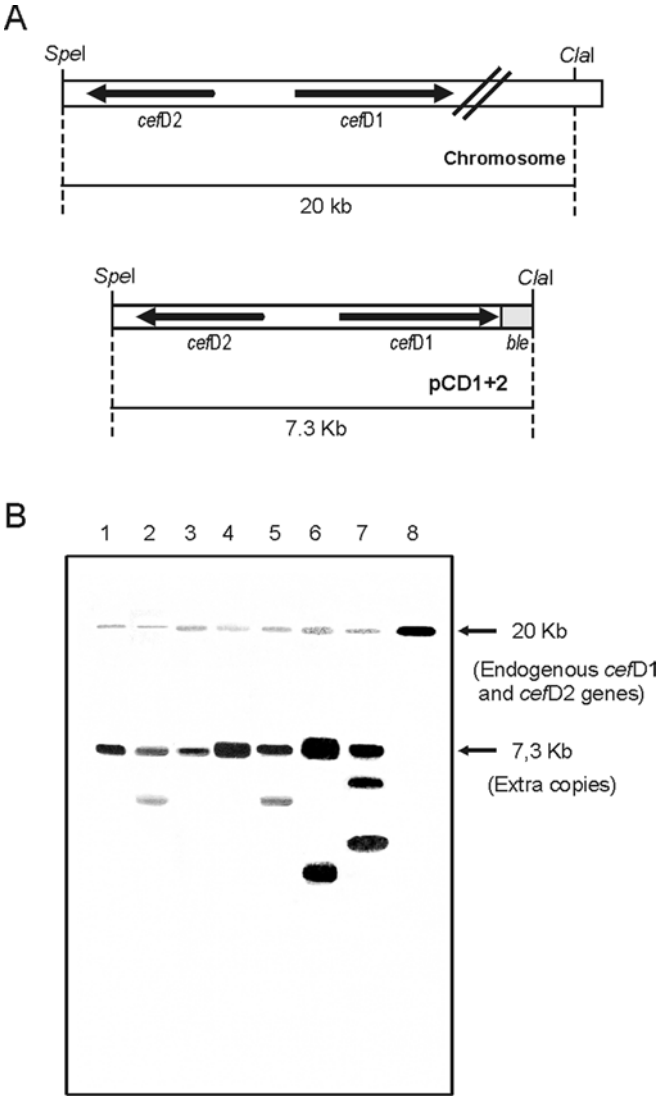

Fig. 1A, B Amplification of the *cefD1-cefD2* region in *A. chrysogenum*. **A** The *Spe* I-*Cla*I fragment containing the *cefD1* and *cefD2* genes in the chromosome and in the plasmid pCD1 + 2. **B** Hybridization analysis of the *A. chrysogenum* C10 strain (lane 8) and transformants (lanes 1–7) containing the *cefD1-cefD2* insert, using as probe the 1.0-kb *Eco* RI-*Hin*dIII fragment of the bidirectional promoter. Note that, whereas the endogenous *cefD1-cefD2* cluster is located on a 20-kb *Spe* I-*Cla*I fragment, the additional copies are located on a 7.3-kb *Spe* I-*Cla*I fragment, as in pCD1 + 2. Lanes: 1, TCMCD2; 2, TMCD26; 3, TCD32; 4, TMCD39; 5, TMCD53; 6, TMCD242; 7, TMCD474; 8, *A. chrysogenum* C10. The number of additional copies was estimated from the ratio of the intensity of the 7.3-kb band to that of the endogenous 20-kb band (single copy). About five times more DNA was loaded in lane 8 (control *A. chrysogenum* C10)

Table 1 Isopenicillin N epimerase activity of *A. chrysogenum* transformants with different numbers of copies of the *cefD1* and *cefD2* genes

Strain[a]	Number of additional copies of *cefD1* and *cefD2*	Epimerase activity (units/mg of protein)[b]
A. chrysogenum C10	0	93 ± 0.94
A. chrysogenum TMCD2	3–4	0
A. chrysogenum TMCD26	2–3	223 ± 15.5
A. chrysogenum TMCD32	3–4	0
A. chrysogenum TMCD39	4–5	0
A. chrysogenum TMCD53	3–4	226 ± 14.5
A. chrysogenum TMCD242	6–7	234 ± 9.5
A. chrysogenum TMCD474	4–5	202 ± 8.2

[a]The strains were grown in DP medium and mycelia were collected at 72 h
[b]Three different clones from each strain were assayed with similar results. The mean values (± SD) are given

Eco RI-*Hin*dIII DNA fragment of the bi-directional *cefD1-cefD2* promoter region. This probe was used in preference to probes internal to *cefD1* or *cefD2* since the latter resulted in hybridization with some heterologous DNA bands in addition to the homologous sequence. Four transformants with higher cephalosporin production than *A. chrysogenum* C10 were randomly selected (TMCD26, TMCD53, TMCD242 and TMCD474). Three transformants with very low cephalosporin production (TMCD2, TMCD32 and TMCD39) were also selected at random. The genomic DNA from these seven transformants and from the parental *A. chrysogenum* C10 strain was digested with *Cla*I + *Spe*I, blotted onto a nylon membrane and hybridized with the labeled probe. The genomic *cefD1* and *cefD2* genes are located on a 20-kb *Cla* I-*Spe*I DNA fragment (Fig. 1A), while the insert in pCD1 + 2, containing intact extra copies of *cefD1* and *cefD2*, gives rise to a 7.3-kb *Cla* I-*Spe*I band. To determine the number of intact additional copies of the *cefD1* and *cefD2* genes in each transformant, the intensity of the 20-kb genomic hybridization band (present as a single copy, as reported previously by Gutiérrez et al. 1999) was compared with that of the 7.3-kb species (Fig. 1B) using a Phosporimager Scanner. This analysis revealed that the number of intact extra copies of *cefD1* and *cefD2* present in these transformants ranged from 2–3 in transformant TMCD26 to 6–7 in TMCD242 (Fig. 1B, Table 1). The hybridization results showed that all the non-producers have a very similar pattern of integration, giving a single hybridizing band of 7.3 kb, whereas in the high producers there were additional hybridizing bands. This result is consistent with integration of the transforming DNA at a preferred site in the genome (see Discussion).

Transformants TMCD26, TMCD53, TMCD242 and TMCD474 overproduce cephalosporins

In order to study the effect of the additional copies of *cefD1* and *cefD2* on cephalosporin production, cultures of the transformants TMCD26, TMCD53, TMCD242, TMCD474, TMCD2, TMCD32 and TMCD39, and the control *A. chrysogenum* C10, were grown in DP medium. The results of three different experiments, each using triplicate flasks, showed (Fig. 2) that transformants TMCD26, TMCD53, TMCD242, and TMCD474 produce about 80-100% more cephalosporin than

Fig. 2A–C Growth (**A**) and cephalosporin production (**B**, **C**) of seven transformants bearing different numbers of copies of the *cefD1-cefD2* region, as compared to the parental *A. chrysogenum* C10 strain (*filled circles*). Note that there is a group of transformants, comprising TMCD26 (*filled inverted triangles*), TMCD53 (*open squares*), TMCD242 (*filled diamonds*) and TMCD474 (*open diamonds*), that show normal growth and an approximately 100% increase in cephalosporin production. The other transformants, TMCD2 (*open circles*), TMCD32 (*open inverted triangles*) and TMCD39 (*filled squares*), showed slower growth and produced almost no cephalosporin. The symbols for the latter three transformants overlap in panels **B** and **C** (no production)

Table 2 HPLC analysis of deacetylcephalosporin C (DAC) and cephalosporin C (CPC) production in *A. chrysogenum* transformants carrying extra copies of the *cefD1* and *cefD2* genes

Strain[a]	DAC production at 120 h (μg of DAC/mg dry weight)	CPC production at 120 h (μg of CPC/mg dry weight)[b]
A. chrysogenum C10	11 ± 0.26	67 ± 1.55
A. chrysogenum TMCD2	0	0.14 ± 0
A. chrysogenum TMCD32	0	0.14 ± 0
A. chrysogenum TMCD39	0	0.17 ± 0
A. chrysogenum TMCD39	0	0.17 ± 0
A. chrysogenum TMCD26	48 ± 1.58	115 ± 3.74
A. chrysogenum TMCD53	48 ± 1.80	116 ± 4.31
A. chrysogenum TMCD242	50 ± 1.76	111 ± 3.89
A. chrysogenum TMCD474	51 ± 1.90	121 ± 4.49

[a]The strains were grown in DP medium and samples were taken at 96 and 120 h
[b]Three different clones from each strain were assayed with similar results. The data shown are mean values (± SD)

A. chrysogenum C10, determined either as volumetric or as specific cephalosporin titers. There was no strict correlation between copy number and the magnitude of the increase in cephalosporin yields. Two or three copies of the *cefD1-cefD2* clusters were sufficient to induce an increase of 100% in the cephalosporin titers, and no further increase in antibiotic production was observed with higher copy numbers, suggesting that a positive transcriptional factor may be titrated out by a high number of copies of the bidirectional *cefD1-cefD2* promoter region. HPLC analysis showed (Table 2) that transformants TMCD26, TMCD53, TMCD242 and TMCD474 produce cephalosporin C and deacetylcephalosporin C in higher concentrations than does the parental strain *A. chrysogenum* C10, suggesting that the amplification effect resulting in higher DAC and cephalosporin C concentration occurs before DAC formation, i.e. at the epimerization stage.

On the other hand, transformants TMCD2, TMCD32 and TMCD39 showed a drastic reduction in

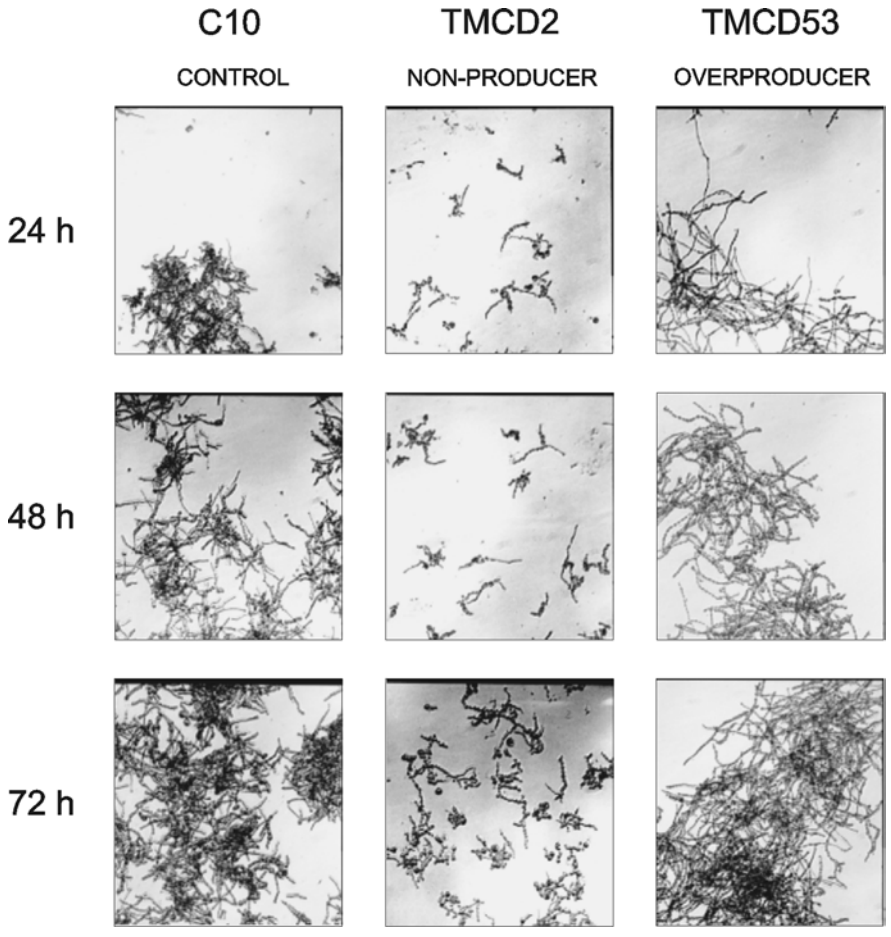

Fig. 3 Mycelium of *A. chrysogenum* C10, the poor cephalosporin-producing transformant TMCD2 and the high producer TMCD53 after 24 h, 48 h and 72 h of culture in DPM medium. Note the unbranched mycelium of transformant TMCD2, which is associated with a disperse growth habit. The other non-producer mutants TMCD32 and TMCD39 exhibited the same morphology TMCD2

cephalosporin production as compared with *A. chrysogenum* C10, as expected from initial tests on solid medium. These three transformants contained 3–5 copies of the intact *cefD1* - *cefD2* cluster, and the lack of cephalosporin production is not due to the absence or rearrangement of the *cefD1-cefD2* cluster, since its organization is not modified. This set of low or non-producing transformants also showed reduced growth in liquid culture (Fig. 2). However, the reduction in growth rate does not in itself explain the lack of cephalosporin production (see below). HPLC analysis revealed that cephalosporin C or deacetylcephalosporin C was barely detectable in culture broths of the transformants TMCD2, TMCD32 and TMCD39.

Transformants TMCD2, TMCD32 and TMCD39 show abnormal mycelial growth

Cephalosporin biosynthesis has been reported to be linked to mycelial differentiation, particularly with the formation of swollen "anthrospore-like" cells (Nash and Huber 1971). To determine whether there is any problem in mycelial differentiation, the mycelia of the different TMCD transformants growing in liquid culture were examined microscopically every 24 h. The cephalosporin-producing transformants TMCD26, TMCD53, TMCD242 and TMCD474 showed a similar mycelial morphology to *A. chrysogenum* C10 (Fig. 3). The mycelia of these strains showed frequent branching and grew as loose pellets. Later, the mycelium formed irregularly swollen cells (arthrospores). However, as shown in Fig. 3, transformants TMCD2, TMCD32 and TMCD39 showed abnormal mycelial growth, forming disaggregated filaments that did not branch like the normal mycelium from *A. chrysogenum* C10. This non-branched mycelium grew as thin filaments and did not form arthrospore-like cells (see Discussion).

Cephalosporin-overproducing transformants show higher levels of IPN epimerase activity than *A. chrysogenum* C10

Determination of the IPN epimerase activity in the different transformants was of great interest, since the increased copy numbers of *cefD1* and *cefD2* might lead to high levels of the IPN epimerase complex or, alternatively, expression of the amplified genes might be limited by the availability of specific *trans* -acting enhancers.

The IPN epimerase activity was measured in different transformants producing high or low levels of cephalosporin, and compared with the IPN epimerase levels found in the parental *A. chrysogenum* strain C10. The results (Table 1) showed that transformants TMCD26, TMCD53, TMCD242 and TMCD474 display higher levels of IPN epimerase activity than *A. chrysogenum* C10, while the poor producers TMCD2, TMCD32, and TMCD39 showed no (or barely detectable) epimerase activity. This result supports the conclusion that the increase in epimerase activity correlates with the rise in cephalosporin production observed in transformants TMCD26, TMCD53, TMCD242 and TMCD474.

The low-producing transformants TMCD2 and TMCD32 show normal levels of IPN but only trace amounts of PenN

To determine if modification of the epimerase activity in TMCD transformants affects the relative levels of IPN

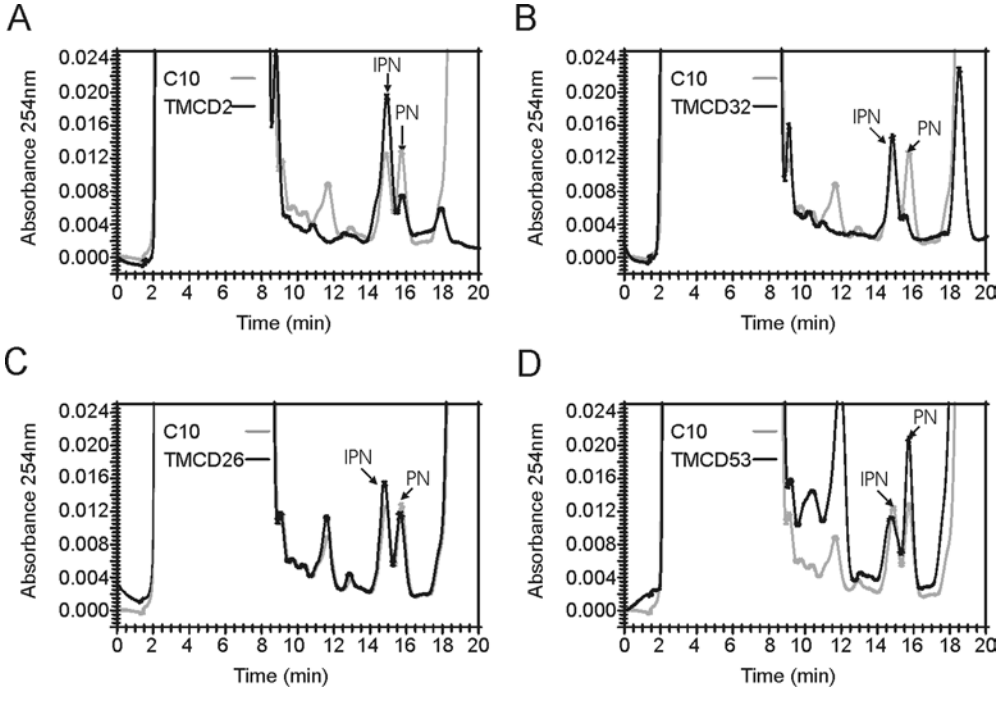

Fig. 4A–D Levels of isopenicillin N (IPN) and penicillin N (here PN) resolved by reverse-phase (C18) HPLC after GITC derivatization (see text). Note that the two non-producing transformants are very inefficient converters of IPN to PenN (**A**, **B**), whereas the high cephalosporin-producing transformants (**C**, **D**) produce both IPN and PenN. PenN accumulates to high levels in transformant TMCD53 (**D**). The curve shown in *gray* indicates the HPLC profile observed for the culture broth of the parental *A. chrysogenum* C10 strain

and PenN, HPLC analysis was performed on culture broths obtained from the transformants TMCD2, TMCD32, TMCD26 and TMCD53, and from *A. chrysogenum* C10 grown for 96 h in DP medium. The results (Fig. 4) showed that strains TMCD2 and TMCD32 produce only small amounts of PenN as compared with *A. chrysogenum* C10, although they produce normal levels of IPN (Fig. 4A and B). This observation correlates well with the lack of IPN epimerase activity. On the other hand, those transformants that overproduced cephalosporin, i.e., TMCD26 and TMCD53 (Fig. 4C and D), showed levels of IPN and PenN similar to those of the parental *A. chrysogenum* C10. It is interesting that transformant TMCD53 accumulated very high levels of PenN, suggesting that this transformant, which expresses a very large amount of IPN epimerase activity (Table 1), now has a bottleneck in the last steps of the cephalosporin pathway.

Transformants impaired in cephalosporin production do not express the *cefD2* gene

The low-producing transformants do not express epimerase activity, despite the fact that they harbour a greater number of intact copies of *cefD1-cefD2*. To gain further insight into the molecular mechanism underlying this lack of epimerase activity, the expression of three genes of the cephalosporin pathway (*pcbC*, *cefD1* and *cefD2*) was investigated by Northern analysis. The results (Fig. 5) showed that the *pcbC* and *cefD1* genes are expressed with normal efficiency, but the *cefD2* gene is not transcribed, even at the basal levels found in the untransformed *A. chrysogenum* C10 prior to 72 h. The *cefD2* gene begins to be expressed some time later at a level close to that seen in the untransformed C10 strain (see Discussion). These results suggest that the expression of *cefD2* is dependent upon a transcriptional activator that is probably defective in the low or non-producing transformants.

It is interesting to note that there is a very good correlation between the increase in *cefD1* and *cefD2* transcript levels in transformant TMCD53 up to 72 h (Fig. 5) and the high levels of IPN epimerase and cephalosporin production in this transformant.

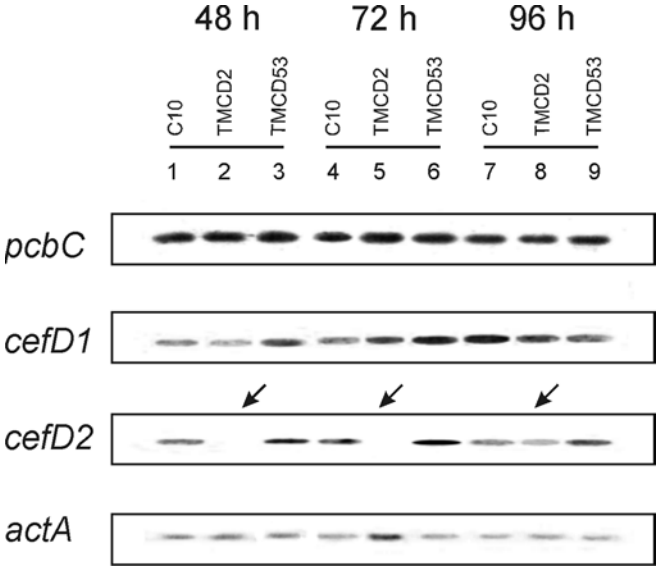

Fig. 5 Northern hybridization analysis of total RNA from *A. chrysogenum* C10 (lanes 1, 4 and 7), TMCD2 (lanes 2, 5, 8) and TMCD53 (lanes 3, 6 and 9) with *pcbC*, *cefD1* , *cefD2* and *actA* (*β*-actin) probes. Note that there is essentially no expression of the *cefD2* gene (*inclined arrows*) in the TMCD2 transformant during the first 72 h of culture (although a significant level of expression was observed at 96 h), whereas the level of the *cefD2* transcript in the overproducing transformant TMCD53 is high

Discussion

Knowledge of the limiting steps in cephalosporin biosynthesis is very important for the development of new strains with improved cephalosporin production (Skatrud et al. 1989; Rodríguez-Sáiz et al. 2004). Many attempts have been made to construct genetically manipulated fungal strains with improved *β*-lactam antibiotic production, but interpretation of the results has been hampered by the lack of basic knowledge about the real rate-limiting steps in the process. It has been reported that ACV synthetase could be a rate-limiting step in penicillin biosynthesis in *Aspergillus nidulans* (Kennedy and Turner 1996). However, the results for *P. chrysogenum,* a fungus that is able to produce much higher levels of penicillin than *A. nidulans*, were rather different. Díez et al. (1989) found only a small increase in penicillin biosynthesis upon amplification of both *pcbC* and *penDE* genes. Similar results were obtained after overexpression of *pcbC* or *penDE* genes under the control of *alcAp* in *A. nidulans* (Fernández-Cañón and Peñalva 1995).

In *A. chrysogenum* there is a bottleneck in the acetylation of DAC to form CPC, the last step in the cephalosporin pathway, which is catalyzed by the DAC acetyltransferase encoded by the *cefG* gene (Gutiérrez et al. 1992). The endogenous *cefG* promoter is very weak, driving the synthesis of very low levels of the *cefG* transcript as compared to the transcription levels of the other cephalosporin biosynthesis genes. Gutiérrez and coworkers (1997) expressed this gene under the control of different promoters and showed that overexpression of the *cefG* gene in *A. chrysogenum* results in a 2- to 3-fold increase in cephalosporin production.

The recently characterized IPN epimerization system in *A. chrysogenum* (Ullán et al. 2002) involves the concerted action of at least two different enzymes, which are required to convert the IPN into PenN, namely an isopenicillin N-CoA synthetase (encoded by *cefD1*) and an isopenicillin N-CoA epimerase (encoded by *cefD2*). Finally, this system may also involve a non-specific acyl-CoA thioesterase that releases the PenN from its CoA

derivative (Fig. 1), although no gene encoding such an enzyme has yet been reported (Martín et al. 2004).

In this work we have obtained a large number of transformants carrying additional copies of both the *cefD1* and *cefD2* genes. In some transformants there is a clear increase in cephalosporin production as compared with the parental *A. chrysogenum* C10, an improved strain from PanLabs (Ramos et al. 1986). These transformants have a higher (about two-fold) IPN epimerase activity than *A. chrysogenum* C10. One of them, TMCD53, was studied in detail and showed an increased content of both *cefD1* and *cefD2* transcripts. Differentiation of *A. chrysogenum* into arthrospores correlates with the cephalosporin production phase (Nash and Huber 1971), although this correlation has not been studied further (Martín and Demain 2002). On the other hand, we obtained many transformants with drastic reductions in cephalosporin biosynthesis that were defective in mycelial branching. This drastic reduction in the amounts of final product following gene amplification is striking, but not unprecedented in filamentous fungi (Verdoes et al. 1994).

The abnormal mycelial development is not due to toxicity of the IPN epimerase resulting from the extra copies of the *cefD1* and *cefD2* genes, since no IPN epimerase activity was detectable in these TMCD transformants. An alternative hypothesis is that there is a titration effect on an inducer due to the high number of promoter copies introduced. However, there is no correlation at all between the number of extra copies in the transformants and IPN epimerase or cephalosporin production levels. Most probably, the problem of mycelial growth is related to the locus into which the exogenous DNA in the genome of the transformants is integrated. The integration did not affect the endogenous *cefD* gene clusters, as demonstrated by Southern analysis, but ectopic integration probably occurs preferentially in a site that is important for growth and secondary metabolism. This hypothesis is consistent with the observed hybridization pattern, which shows a single hybridizing band in these transformants. Although the endogenous cephalosporin clusters should still be functional, IPN epimerase activity was barely detectable and, therefore, no DAC or CPC was formed.

The results of the transcription studies provided evidence indicating that *pcbC* and *cefD1* are still expressed in the non-producing transformants, whereas transcription of *cefD2* does not occur prior to 72 h of incubation. It is interesting to note that even the expression of the endogenous *cefD2* gene is prevented in these transformants (compare lanes 1 and 2, or 4 and 5 in Fig. 5). All the evidence indicates that a transcriptional factor required for the expression of *cefD2* has been knocked out as a result of preferential integration at a specific locus in the non-producer mutants, thus preventing expression of both the endogenous and the extra copies of the *cefD2* gene.

In this work we have also found that the epimerization step is a rate-limiting step in cephalosporin biosynthesis in *A. chrysogenum*. We have shown that when the epimerase activity is enhanced in *A. chrysogenum* there is a clear increase in cephalosporin production. Thus, this method provides a strategy that could easily be applied to improve industrial cephalosporin producing strains.

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