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The unprocessed preprotein form IAT^{C103S} of the isopenicillin *N* acyltransferase is transported inside peroxisomes and regulates its self-processing ☆

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ABSTRACT

Previous studies in *Penicillium chrysogenum* and *Aspergillus nidulans* suggested that self-processing of the isopenicillin *N* acyltransferase (IAT) is an important differential factor in these fungi. Expression of a mutant *penDE*^{C103S} gene in *P. chrysogenum* gave rise to an unprocessed inactive variant of IAT (IAT^{C103S}) located inside peroxisomes, which indicates that transport of the proIAT inside these organelles is not dependent on the processing state of the protein. Co-expression of the *penDE*^{C103S} and wild-type *penDE* genes in *P. chrysogenum* (Wis54-*DE*^{C103S} strain) led to a decrease in benzylpenicillin levels. Changes in the wild-type IAT processing profile (β subunit formation) were observed in the Wis54-*DE*^{C103S} strain, suggesting a regulatory role of the unprocessed IAT^{C103S} in the processing of the wild-type IAT. This was confirmed in *Escherichia coli*, where a delay in the processing of IAT in presence of the unprocessable IAT^{C103S} was observed. Our results indicate that IAT is post-translationally regulated by its preprotein, which interferes with the self-processing.

1. Introduction

Penicillium chrysogenum, *Aspergillus nidulans* and a few other ascomycetes (Aharonowitz et al., 1992; Laich et al., 2003) are filamentous fungi able to synthesize penicillins containing an aromatic side chain (e.g., benzylpenicillin or phenoxymethylpenicillin). Penicillins are synthesized by internal cyclization of the non-ribosomal tripeptide δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) formed by the multienzyme δ(α-aminoadipyl)-cysteinyl-valine synthetase (encoded by the *pcb*AB gene). This tripeptide is cyclized to isopenicillin N by the action of isopenicillin N synthase (encoded by the *pcb*C gene) (Martín and Liras, 1989). In the last step, the isopenicillin *N* acyltransferase (IAT; encoded by the *penDE* gene) synthesizes hydrophobic penicillins by substitution of the L-α-aminoadipyl side chain of isopenicillin N by aromatic acyl side chains (Álvarez et al., 1987; Martín et al., 1990). The aromatic (phenylacetic or phenoxyacetic) acid requires previous activation by a specific aryl-CoA ligase (Lamas-Maceiras et al., 2006). In *P. chrysogenum*, the *pcbAB*, *pcbC* and *penDE* genes are clustered with other ORFs forming an amplifiable DNA unit, which is present in several copies in high penicillin producing strains (Fierro et al., 2006). Each unit is bound by a conserved hexanucleotide TGTAA$^{A}/_{T}$ (Fierro et al., 1995). The *penDE* genes, cloned from *P. chrysogenum* (Barredo et al., 1989) and *A. nidulans* (Montenegro et al., 1990; MacCabe et al., 1991), are very similar in both fungi.

The IAT is synthesized as a 40-kDa precursor (proacyltransferase, proIAT) which undergoes an autocatalytic self-processing between residues Gly102-Cys103 in *P. chrysogenum*. The processed protein constitutes a heterodimer with subunits α (11 kDa, corresponding to the N-terminal fragment) and β (29 kDa, corresponding to the C-terminal region) (Barredo et al., 1989; Veenstra et al., 1989; Whiteman et al., 1990; Tobin et al., 1990, 1993). Unlike the two first enzymes involved in penicillin biosynthesis, which are cytosolic, IAT is located inside the microbodies (peroxisomes) (Müller et al., 1991, 1992, 1995). Although it was initially believed that the IAT activity was located in the 29-kDa subunit (Álvarez et al., 1987), later it was shown that a higher IAT activity is observed after association of the 29-kDa and 11-kDa subunits (Tobin et al., 1990, 1993), indicating that the most active *P. chrysogenum* IAT is an α–β heterodimer (Whiteman et al., 1990). More recently we found that the *A. nidulans* proIAT remains predominantly unprocessed as a 40-kDa protein through several purification steps, whereas the *P. chrysogenum* enzyme is efficiently self-processed, rapidly dissociating into the 29-kDa and 11-kDa subunits (Fernández et al., 2003). In fact, only the 29-kDa subunit (but never the 40-kDa unprocessed form) is isolated from *P. chrysogenum*

☆ Regulation of IAT processing in *P. chrysogenum*.

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crude extracts and purified fractions (Álvarez et al., 1987; Fernández et al., 2003), suggesting that an efficient self-processing is associated with high rates of penicillin biosynthesis. In *A. nidulans* a small amount of both the 29 and 11 kDa subunits was observed but the 40 kDa unprocessed form was always found (Fernández et al., 2003). This difference in IAT processing ability between *P. chrysogenum* and *A. nidulans* might be responsible (at least in part) for the disparity in penicillin biosynthetic ability between these two fungi (wild-type strains of *P. chrysogenum* are able to synthesize about 30-fold higher levels of penicillin than wild-type *A. nidulans*).

Many fungal enzymes are formed as proenzymes (Markaryan et al., 1996) and the mechanism of self-processing is frequently a step involved in post-translational regulation of enzyme activity (Park et al., 2004). The *P. chrysogenum* IAT is an N-terminal nucleophile (NTN) enzyme capable of self-activation (J.F. Martín and C. García-Estrada, unpublished results), as it occurs with other NTN amidohydrolases (Brannigan et al., 1995; Seemüller et al., 1996).

One of the best known NTN enzymes is the *Escherichia coli* penicillin acylase (Duggleby et al., 1995). Hewitt and co-workers (2000) reported evidence indicating that in a slow-processing mutant of the *E. coli* penicillin proacylase, the linker peptide located between the α and β subunits, blocks the active site cleft of this enzyme.

At present it is unknown whether the self-processing IAT from *P. chrysogenum* works by a mechanism similar to that of other NTN enzymes and whether unprocessable proIAT or slow-processing forms, may regulate the self-processing activity of the IAT. It was, therefore, important to obtain an unprocessable mutant IAT and to construct strains in which the unprocessable IAT is synthesized together with the native processable IAT, to establish the *in vivo* role of unprocessed molecules on IAT activity and penicillin biosynthesis.

Tobin and co-workers (1995) showed that the recombinant IAT^{C103S} is not self-processed in *E. coli* and lacks acyltransferase activity. We report in this article, new insights on the IAT of *P. chrysogenum* including the *in vivo* characterization and targeting of the unprocessed IAT^{C103S} variant and the construction of *P. chrysogenum* strains, which simultaneously synthesize the wild-type IAT and an unprocessed variant of the protein. Results provide evidence that the unprocessed proIAT is correctly targeted to peroxisomes and affects the benzylpenicillin biosynthesis through regulation of the self-processing.

2. Materials and methods

2.1. Strains, culture conditions, plasmid constructs and transformation of P. chrysogenum protoplasts

The strains and plasmids used in this work are listed in Table 1. Fungal spores were plated on Power medium (Casqueiro et al., 1999) and grown for 5 days at 28 °C. *P. chrysogenum* liquid cultures were initiated by inoculating fresh spores in 100 ml of DP medium (Casqueiro et al., 1999) without phenylacetate. After incubation at 25 °C for 20 h in an orbital shaker (250 rpm), a 10 ml aliquot was inoculated in 100 ml DP medium with 1 mg/ml phenylacetate (in 500-ml flasks). Protoplasts were obtained and transformed as previously described (Cantoral et al., 1987; Díez et al., 1987). Selection of transformant clones was performed by resistance to phleomycin (30 μg/ml). *E. coli* JM109 (DE3) cells were used for heterologous expression of the *penDE* and *penDE^{C103S}* genes and were grown in Luria–Bertani (LB) medium supplemented with the appropriate antibiotic.

2.2. Site-directed mutagenesis of the penDE gene

Plasmids pPenIAT (García-Estrada et al., 2007) and pPBCαβ (Fernández et al., 2003) (Table 1), which contain the cDNA of the *penDE* gene, were used as template for site-directed mutagenesis of the Cys103 to Ser using the "*QuickChange® Site-Directed Mutagenesis Kit*" (Stratagene La Jolla, CA, USA) following the manufacturer's instructions. Primers used to introduce this mutation were purified by HPLC; 5′-CAGCCCGTGATGGCTCCACCACTGCCTATTG-3′ (forward), 5′-CAATAGGCAGTGGTGGAGCCATCACGGGCTG-3′ (reverse) (the triplet corresponding to the mutated Cys103 to Ser is underlined). The point mutation was confirmed by sequencing and the resulting plasmids were named pPenIAT^{C103S} and p*DE^{C103S}*, respectively (Table 1).

2.3. Genomic DNA extraction and Southern blotting

DNA isolation and Southern blotting hybridisation were carried out as previously described (Lamas-Maceiras et al., 2006). A 429-bp fragment, spanning positions 727-1155 of the *penDE* gene (inside the third exon), was used as probe. This fragment was amplified using next primers: 5′-CCCACCGGAGTTCCTTCG-3′ (forward)

Table 1
Fungal strains and plasmids used in this work

	Features	Origin
P. chrysogenum strain		
Wis54-1255	Single copy of the penicillin gene cluster	Fierro et al. (1995)
npe10-*AB·C*	Strain derived from the npe10 pyrG$^-$ (Δ*pen*). It contains the first two genes of the penicillin pathway (*pcbAB* and *pcbC*) integrated in the genome	García-Estrada et al. (2007)
npe10-*AB·C·DE*	npe10-*AB-C* strain complemented with the *penDE* gene under the control of the *gdh* gene promoter	García-Estrada et al. (2007) and this work
npe10-*AB·C·DE^{C103S}*	npe10-*AB-C* strain complemented with the *penDE^{C103S}* gene under the control of the *gdh* gene promoter	This work
Wis54-*DE^{C103S}*	Wis54-1255 strain complemented with the *penDE^{C103S}* gene under the control of the *gdh* gene promoter	This work
Plasmids		
pPenIAT	Plasmid used to express the *penDE* gene in *P. chrysogenum*. It contains the cDNA of the *penDE* gene under the control of the *gdh* gene promoter	García-Estrada et al. (2007)
pPenIAT^{C103S}	Similar to pPenIAT, but by site-directed mutagenesis, the cDNA of the *penDE* gene has been mutated to change the Cys103 to Ser in the IAT	This work
pPBCαβ	Plasmid used to express the cDNA of the *penDE* gene in *E. coli*	Fernández et al. (2003)
p*DE^{C103S}*	Similar to pPBCαβ, but by site-directed mutagenesis, the cDNA of the *penDE* gene has been mutated to change the Cys103 to Ser in the recombinant IAT	This work
pαβ-*DE^{C103S}*	Plasmid used to co-express simultaneously both the *penDE* and the *penDE^{C103S}* genes in *E. coli* The BamHI–KpnI fragment of pPBCαβ, containing the pT7 and the cDNA of the *penDE* gene was inserted in the same restriction sites of plasmid pQE-30. The SmaI–HindIII fragment of p*DE^{C103S}*, including the pT7 and the cDNA of the pen*DE^{C103S}* gene was subcloned between the SmaI and HindIII restriction sites of plasmid pQE-30	This work

and: 5′-GATGATATTGAACAGAGTC-3′ (reverse). The signal provided by the Southern blotting was quantified by densitometry using the "Gel-Pro Analizer" software (Media Cybernetics). The number of additional copies of the P*gdh*-*penDE*^{C103S}-T*cyc1* cassette was determined comparing the intensity of the 2120-bp band (including the exogenous *penDE*^{C103S} gene) to that of the 8000-bp band (containing the wild-type *penDE* gene present as a single copy in the Wis54-1255 strain (Fierro et al., 1995)).

2.4. Electron microscopy

Mycelia from *P. chrysogenum* Wis54-1255, *P. chrysogenum* npe10-*AB-C* and *P. chrysogenum* npe10-*AB-C-DE*^{C103S} strains were grown for 48 h under benzylpenicillin production conditions as described above. Fixation and preparation of the samples for electron microscopy was performed as described previously (Waterham et al., 1994). Ultrathin sections of unicryl-embedded (BBI International, Cardiff, UK) hyphae were immunolabeled with specific antibodies raised against IAT (1/4000 dilution) and 15 nm gold-conjugated goat-anti-rabbit antibodies (Amersham Biosciences Corp., Piscataway, NJ, USA).

2.5. Cell extracts

Penicillium chrysogenum mycelia were centrifuged for 10 min at 4400 rpm, washed three times in 0.9% (w/v) NaCl and powdered in a grinder using liquid nitrogen. The disrupted cells were resuspended in TD buffer (50 mM Tris–HCl, pH 8.0, 5 mM DTT) and stored at −80 °C. *E. coli* cells were harvested by centrifugation at 4400*g* for 10 min, washed with 0.9% (w/v) NaCl and resuspended in TD buffer. Cells were broken after sonication on ice (6 pulses of 10 s with 30 s pauses between each pulse) and centrifuged at 4400*g* for 10 min at 4 °C. Soluble fractions were stored at −80 °C.

2.6. IAT self-processing assessment in E. coli

Self-processing of wild-type IAT either in absence or presence of the unprocessed IAT^{C103S} was tested in competent *E. coli* JM109 cells. They were transformed with plasmid pPBCαβ (which expresses only the wild-type *penDE* gene) or with plasmid pαβ-*DE*^{C103S} (which expresses both the wild-type *penDE* and the *penDE*^{C103S} genes as indicated in Table 1). Simultaneous cultures were inoculated and induced with 0.5 mM IPTG at 37 °C for 45 min to provide enough amounts of the 40 kDa protein (only the wild-type protein able to be properly folded in cells transformed with plasmid pPBCαβ and a mixture of wild-type IAT and IAT^{C103S} variant in cells transformed with plasmid pαβ-*DE*^{C103S}). Cells were washed three times with 0.9% NaCl to remove the IPTG, resuspended in LB media and incubated at 26 °C. These conditions allow the self-processing of the preprotein. This artificial processing system provided us with a useful tool to compare the processing of IAT in absence and presence of the IAT^{C103S}. One milliliter samples were taken at different time points, centrifuged, resuspended in 100 μl of loading buffer (see below) and diluted 1000-fold. Then, they were run in SDS–PAGE gels and analyzed by Western blotting.

2.7. SDS–PAGE and Western blotting

Protein samples to be analysed by SDS–PAGE were diluted in loading buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol and 0.1% bromophenol blue), boiled for 5 min, and run in a 12% acrylamide gel. The "Low Molecular Weight Calibration Kit for SDS Electrophoresis" (Amersham Biosciences Corp.) and "Precision Plus Protein All Blue Standards" (Bio-Rad, Hercules, CA, USA), were used as molecular weight marker. Proteins were stained using Coomassie Brilliant Blue R250 dying. Immunological detection of IAT (wild-type and IAT^{C103S} mutant) was performed as previously described (García-Estrada et al., 2007). Polyclonal antibodies against *P. chrysogenum* IAT were obtained as indicated before (Fernández et al., 2003).

2.8. In vitro IAT activity measurement

Measurement of the IAT activity was carried out using *E. coli* soluble extracts (see above). Briefly, 36 μl of cell extracts containing the recombinant IAT were mixed either with 36 μl of soluble extract containing the recombinant unprocessable IAT^{C103S}, with 36 μl of 1/10 dilution of soluble extract containing the recombinant unprocessable IAT^{C103S} or with 36 μl of uninduced *E. coli* extract (as control). Extracts were pre-incubated for 1 and 5 h at 26 °C. After the pre-incubation time, 48 μl of the reaction mixture (0.1 M Tris–HCl, pH 8.0, 0.05 M DTT, 0.2 mM 6-APA and 0.2 mM phenylacetyl-CoA) were added and incubated at 26 °C for 15 min. The reaction was stopped with 120 μl of methanol, centrifuged at 13,000 rpm for 5 min and bioassayed. *In vitro* reactions were performed in triplicates.

2.9. RNA extraction and RT-PCR assays

RNA was extracted from cultures of *P. chrysogenum* at 24, 48 and 72 h using the "RNeasy Mini Kit" columns (Qiagen, Hilden, Germany), following the manufacturer's instructions. Total RNA was treated with "RQ1 RNase-Free DNase" (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions and quantified using a NanoDrop ND-1000 spectrophotometer. RT-PCR was performed using 50 ng of total RNA and the "SuperScript One-Step RT-PCR with Platinum Taq" system (Invitrogen Corporation, Carlsbad, CA, USA), following the manufacturer's instructions. Reverse primer was specific to the initial part of the T*cyc1* terminator: 5′-GAAAAGGGGGACGGATCTCCG-3′. Forward primer included the start codon of the *penDE* gene: 5′-ATGCTTCACATCCTCTGTC AAGG-3′. Annealing temperature was set to 55 °C during 35 cycles.

2.10. Bioassays and HPLC analysis

Filtrates from *P. chrysogenum* cultures on liquid DP medium (Casqueiro et al., 1999) or samples obtained in the *in vitro* acyltransferase activity experiments, were used to assess the formation of isopenicillin N or benzylpenicillin. *Micrococcus luteus* was used as test microorganism. It was grown at OD_{600} = 2 in TSB (Difco) and inoculated into TSA (Difco) medium at a final OD_{600} = 0.01. Benzylpenicillin extraction and analysis by HPLC were carried out as previously indicated (García-Estrada et al., 2007).

3. Results

3.1. In vivo characterization of a tailored P. chrysogenum strain which expresses the mutant penDE^{C103S} gene encoding the unprocessable IAT^{C103S}

The change of Cys103 residue to Ser (C103S) is known to abolish self-processing and activity of IAT in *E. coli* (Tobin et al., 1995). In order to express the mutant *penDE*^{C103S} gene in *P. chrysogenum*, plasmid pPenIAT^{C103S}, in which the mutant *penDE*^{C103S} gene is expressed from the strong *A. awamori gdh* promoter (Table 1), was constructed as indicated in Section 2. This plasmid was introduced into the *P. chrysogenum* npe10-*AB-C* strain, which is able to synthesize isopenicillin N and lacks the *penDE* gene (García-Estrada et al., 2007). Transformants were selected for their ability to grow in medium with 30 μg/ml phleomycin. The presence of the *penDE*^{C103S}

allele was tested by PCR (data not shown) and integration of the P*gdh-penDE*^{C103S}-T*cyc1* cassette was confirmed by Southern blotting (Fig. 1A) using a probe internal to the *penDE* gene. Transformants T22, T24 and T30 showed a 2120-bp band after the digestion of the genomic DNA with HindIII and KpnI. This band corresponds to the whole P*gdh-penDE*^{C103S}-T*cyc1* cassette. Additional bands, which are a result of the integration of fragments of this cassette, are visible in transformants T4, T9, T24 and T30. Expression of the mutant gene was tested by Western blotting using cell extracts obtained from 24-h cultures of the different transformants. As shown in Fig. 1B, the 40-kDa mutant proIAT^{C103S} was generated only in those transformants with a full integration of the P*gdh-penDE*^{C103S}-T*cyc1* cassette (note the lack of self-processing of the IAT^{C103S}). Transformant T22 was selected and was named *P. chrysogenum* npe10-*AB-C-DE*^{C103S}. As internal control of these experiments, plasmid pPenIAT, which contains the cDNA of the *penDE* gene under the control of the strong *A. awamori gdh* gene promoter (García-Estrada et al., 2007), was transformed into the npe10-*AB-C* strain, thus generating the npe10-*AB-C-DE* strain. Expression of this gene generated an IAT capable of self-processing (Fig. 1B) and restored the penicillin production (data not shown), as it was previously reported (García-Estrada et al., 2007).

The ability of the mutant protein to remain unprocessed in *P. chrysogenum* npe10-*AB-C-DE*^{C103S}was tested. As shown in Fig. 1C, only the 40-kDa band is visible throughout the culture. This band is more abundant at 36-48 h and then gradually decreases until 72 h. This shows the lack of processing of the mutant IAT^{C103S} when it is synthesized in *P. chrysogenum* and indicates that no other proteolytic mechanism different from autocatalysis (absent

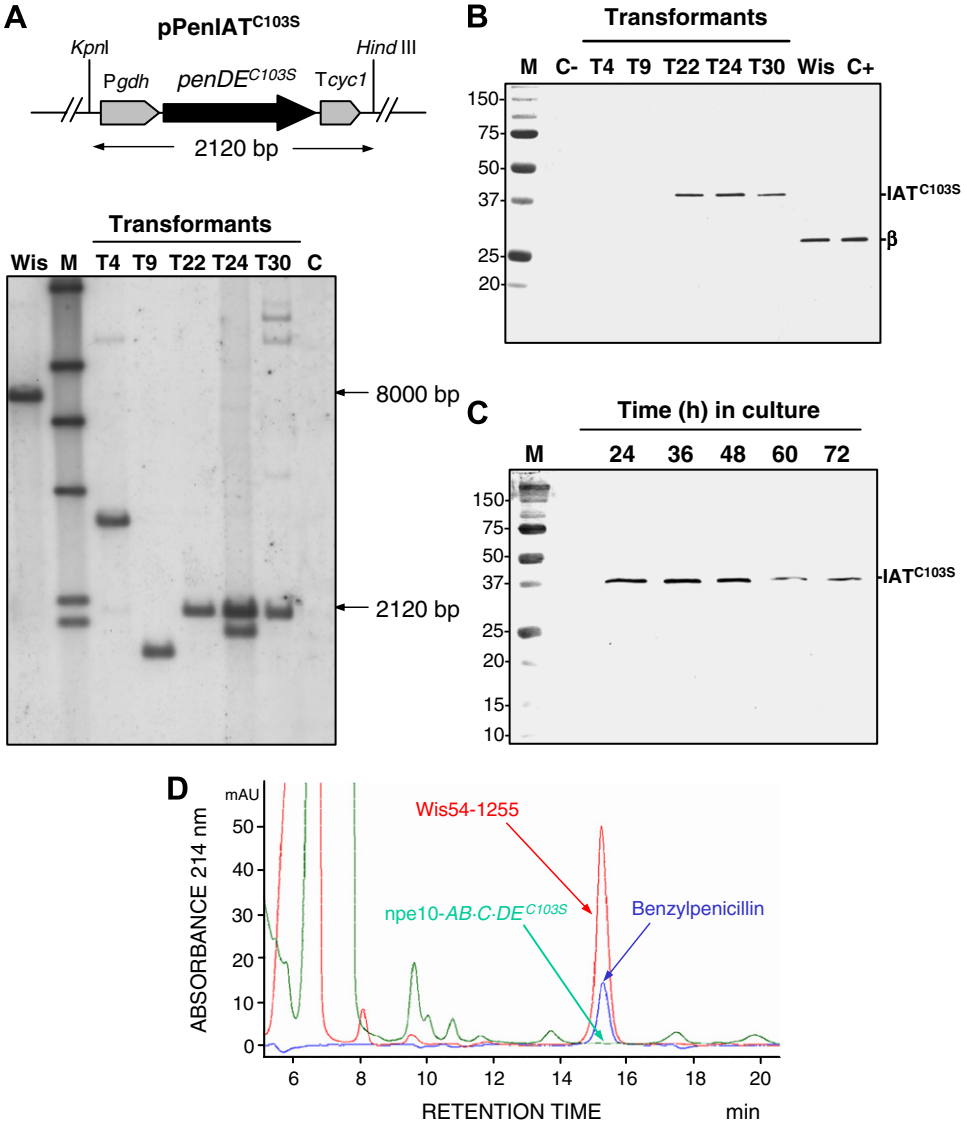

Fig. 1. Expression of the *penDE*^{C103S} gene and activity of the unprocessed IAT^{C103S} in *P. chrysogenum* npe10-*AB-C*. (A) The npe10-*AB-C* strain was transformed with plasmid pPenIAT^{C103S}. Different transformants (T4, T9, T22, T24 and T30) were tested by Southern blotting after digestion of the genomic DNA with HindIII and KpnI, which releases the whole P*gdh-penDE*^{C103S}-T*cyc1* cassette (2120 bp). Bands of different size indicate integration of fragments of the P*gdh-penDE*^{C103S}-T*cyc1* cassette in the transformants. Genomic DNA from the Wis54-1255 strain (Wis), which shows an 8000 bp band (internal *penDE* gene), was used as positive control, whereas DNA from the npe10-*AB-C* strain was used as negative control (C). The λ-HindIII molecular weight markers are indicated as M. (B) Western blot carried out with protein extracts isolated from 24-h cultures of the different transformants and antibodies raised against IAT (they recognize the β subunit and the α–β proIAT but not the separate α subunit). Protein extracts from the npe10-*AB-C* strain were used as negative control (C−), whereas the npe10-*AB-C-DE* (C+) and Wis54-1255 (Wis) strains were used as positive controls. Molecular weight markers (lane M) are indicated on the left in kDa. Transformant T22 was selected and was named *P. chrysogenum* npe10-*AB-C-DE*^{C103S}. (C) Western blot showing the synthesis of the unprocessed IAT^{C103S} in the npe10-*AB-C-DE*^{C103S} strain. Samples were taken at 24, 36, 48, 60 and 72 h, and hybridized with anti-IAT antibodies. (D) HPLC of filtrates taken at 72 h from cultures of the npe10-*AB-C-DE*^{C103S} and the Wis54-1255 strains. As internal control a pure benzylpenicillin sample was used at a concentration of 10 μg/ml.

in the IAT^{C103S} mutant) is involved in the processing of IAT in this fungus.

The benzylpenicillin biosynthetic activity of the IAT^{C103S} was assessed in the *P. chrysogenum* npe10-*AB-C-DE*^{C103S} strain by bioassay (data not shown) and by HPLC using filtrates taken at 72 h from cultures of the npe10-*AB-C-DE*^{C103S} and the Wis54-1255 strains. Chromatograms showed the absence of benzylpenicillin in filtrates from the npe10-*AB-C-DE*^{C103S} strain (Fig. 1D), indicating that the unprocessed IAT lacks enzymatic activity *in vivo*.

3.2. The unprocessed IAT is transported into peroxisomes

An important question is whether the unprocessed IAT can be transported into peroxisomes. To test this, subcellular localization of the IAT^{C103S} was determined by immunocytochemistry as indicated in Section 2. *P. chrysogenum* npe10-*AB-C-DE*^{C103S} (expressing the mutant *penDE*^{C103S}gene), Wis54-1255 (expressing the wild-type *penDE* gene) and npe10-*AB-C* (lacking the *penDE* gene) strains were analysed using electron microscopy. Immunolabeling with antibodies raised against IAT clearly showed that the unprocessed IAT^{C103S} is located on the peroxisomal matrix (Fig. 2A), as occurs with the wild-type protein (Fig. 2B). No significant labeling was obtained with the control npe10-*AB-C* strain (Fig. 2C) since it lacks the *penDE* gene and therefore, IAT is absent. These results indicate that the proIAT form (unprocessed) is able to be transported into peroxisomes, and therefore, self-processing may occur optimally under the internal peroxisomal ambient conditions.

3.3. Effect of the unprocessed IAT^{C103S} on penicillin biosynthesis

Penicillium chrysogenum proIAT is very efficiently self-processed and rapidly dissociated into the 29-kDa and 11-kDa subunits. Therefore, it is very difficult to test whether the unprocessed preprotein is able to exert some *in vivo* regulatory effect in this fungus. To assess this, we transformed the Wis54-1255 strain with plasmid pPenIAT^{C103S}, which expresses the mutant *penDE*^{C103S} gene. This gene gives rise to the unprocessed IAT^{C103S} protein, which is co-localized with the wild-type IAT and lacks acyltransferase activity as seen above. Positive transformants containing both the wild-type (1274-bp genomic DNA) and the mutant *penDE*^{C103S} (1071-bp intronless cDNA) genes were identified by PCR (data not shown). Integration of the P*gdh-penDE*^{C103S}-T*cyc1* cassette into the Wis54-1255 strain was confirmed by Southern blotting (Fig. 3A) in six randomly selected transformants using a fragment of the *penDE* gene as probe. All transformants showed the band with the internal wild-type *penDE* gene (8000 bp) plus a 2120-bp band, which corresponds to the whole P*gdh-penDE*^{C103S}-T*cyc1* cassette. Additional bands, which are a result of the integration of fragments of this cassette, are visible in transformants T7 and T20. Densitometric analysis of the Southern blotting revealed that a different number of copies of the whole cassette had been integrated in the genome of these transformants; 1–2 copies in transformants T4, T7, T21 and T29, and 3–4 copies in transformants T16 and T20. Expression of the *penDE*^{C103S} gene was tested by RT-PCR (Fig. 3B). In order to discriminate between the endogenous wild-type *penDE* transcripts and the exogenous *penDE*^{C103S} mRNAs generated from the P*gdh-penDE*^{C103S}-T*cyc1* cassette, retrotranscription was performed using a reverse primer specific to the 5′ region of the T*cyc1* terminator. An oligonucleotide including the start codon of the *penDE* gene was used as forward primer. All transformants were able to express the *penDE*^{C103S} gene, and were named *P. chrysogenum* Wis54-*DE*^{C103S}. Transformants containing more copies of the P*gdh-penDE*^{C103S}-T*cyc1* cassette (T16 and T20), gave rise to higher steady-state mRNA levels along the culture, especially at 48 and 72 h. The synthesis of both the wild-type IAT and the unprocessable IAT^{C103S} variant was confirmed by Western blotting using protein extracts taken from 24-h cultures of the different transformants (Fig. 3C). Benzylpenicillin specific production was analysed at different time points in the Wis54-1255 strain and transformants of the Wis54-*DE*^{C103S} strain. As shown in Fig. 3D, the Wis54-*DE*^{C103S} transformants produced lower amounts of benzylpenicillin than the Wis54-1255 strain. The decrease in the β-lactam production was especially remarkable in transformants T16 and T20. This difference in penicillin production among the transformants is likely dependent on the integration site of plasmid pPenIAT^{C103S} in the *P. chrysogenum* genome that may facilitate its expression and may also be due to differences in the *penDE*^{C103S} gene copy number. In transformants T16 and T20, there are more copies of the *penDE*^{C103S} gene (Fig. 3A) and levels of the mutant *penDE* transcript were highest (Fig. 3B), which indicates that the biosynthesis of the antibiotic is affected by the presence of the IAT^{C103S} protein.

3.4. The β subunit formation pattern is affected by the IAT^{C103S} in *P. chrysogenum*

In order to find out the mechanism by which the unprocessed IAT^{C103S} is affecting the biosynthesis of benzylpenicillin, the β subunit formation pattern of wild-type IAT was studied in presence (transformants T16 and T20 of the Wis54-*DE*^{C103S} strain) and absence (Wis54-1255 strain) of the mutant IAT^{C103S}. Thus, protein extracts taken from cultures of these strains, were analysed by

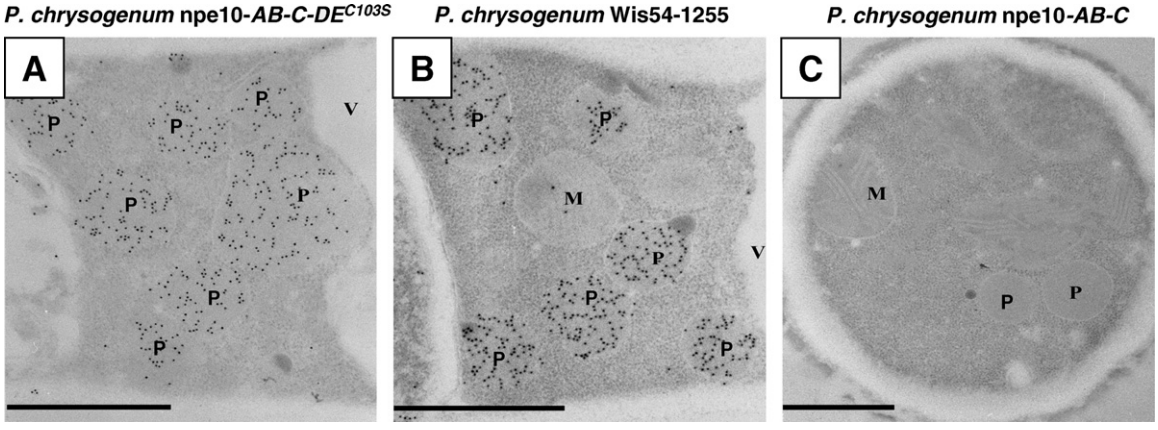

Fig. 2. Immunocytochemical localization of the unprocessed IAT^{C103S}. Electron microscopy pictures of ultrathin sections of unicryl-embedded hyphae from *P. chrysogenum* npe10-*AB-C-DE*^{C103S} (A) Wis54-1255 (B) and npe10-*AB-C* (C) strains immunolabeled with anti-IAT antibodies and gold-conjugated goat-anti-rabbit antibodies. M, Mitochondrion; P, peroxisomes; V, vacuole. Bar represents 1 μm. Note the absence of IAT signal in the control strain and the peroxisomal location of the IAT^{C103S}.

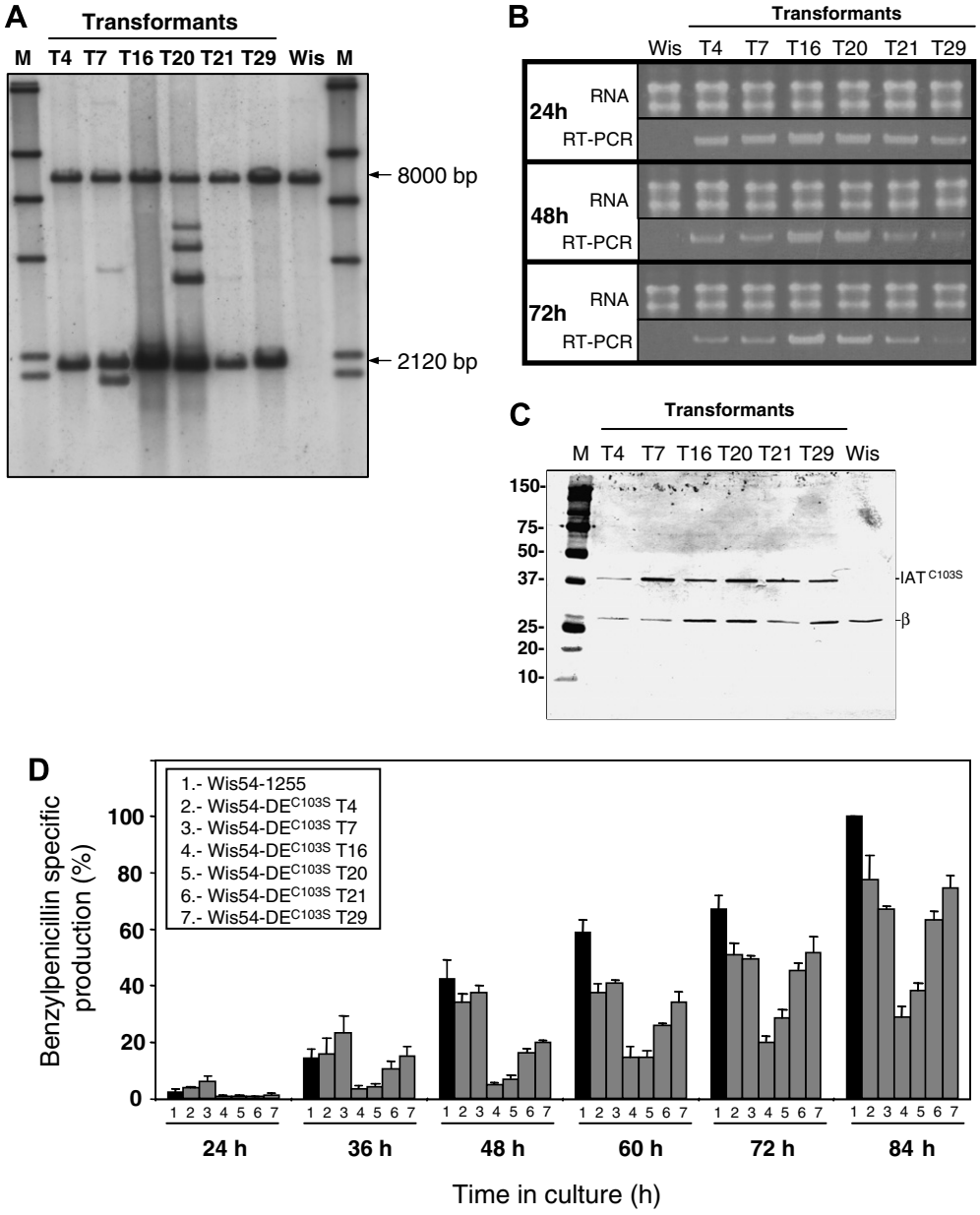

Fig. 3. Characterization of a tailored *P. chrysogenum* strain which synthesizes both the wild-type IAT and the unprocessed variant IAT^{C103S}. (A) The Wis54-1255 strain was transformed with plasmid pPenIAT^{C103S}. Different transformants (T4, T7, T16, T20, T21 and T29) were tested by Southern blotting after digestion of the genomic DNA with HindIII and KpnI, which releases the whole P*gdh*-*penDE*^{C103S}-T*cyc1* cassette (2120 bp) and an 8000-bp band with the internal wild-type *penDE* gene. Bands of different size indicate integration of fragments of the P*gdh*-*penDE*^{C103S}-T*cyc1* cassette in these transformants. Genomic DNA from the Wis54-1255 strain (Wis) was used as positive control. The λ-HindIII molecular weight marker is indicated as M. (B) RT-PCR experiments showing the expression of the *penDE*^{C103S} gene in different transformants of the Wis54-*DE*^{C103S} strain. RNA was extracted at 24, 48 and 72 h from cultures. Retrotranscription was performed using a reverse primer specific to the initial part of the T*cyc1* terminator. An oligonucleotide including the start codon of the *penDE* gene was used as forward primer. The rRNA bands (R) are shown to indicate that similar amounts of RNA been used in the RT-PCR reaction (T). RNA extracted from the Wis54-1255 strain (Wis) was used as negative control. Control PCR reactions were done to confirm the absence of contaminant DNA in the RNA samples (data not shown). (C) Western blot carried out with protein extracts isolated from 24-h cultures of different transformants of the Wis54-*DE*^{C103S} strain, and antibodies raised against IAT. Protein extracts obtained from the Wis54-1255 strain were used as control (Wis). Molecular weight markers (lane M) are indicated on the left in kDa. (D) Benzylpenicillin specific production of Wis54-1255 strain (black bars) and different transformants of the Wis54-*DE*^{C103S} strain (gray bars). Filtrates were taken at different time points from three duplicated cultures and the penicillin was quantified by bioassay. Results are represented as the mean percentage (%) ± SD of the benzylpenicillin specific production of the Wis54-1255 strain at 84 h (set to 100%).

Western blotting. The same amount of protein was loaded in each lane. In the parental Wis54-1255 strain, the β subunit (evidencing rapid proIAT self-processing) is more abundant at 36–48 h, decreasing thereafter (Fig. 4). However, when the unprocessed IAT^{C103S} was present, the pattern of β subunit formation was altered in transformants T16 and T20 (Fig. 4). In these transformants, the 40-kDa protein band is present throughout the culture time. However, the β subunit, originated after processing of the wild-type IAT, is only detected during the initial 24–48 h of culture. Since the antibodies cannot distinguish between the IAT^{C103S} and the wild-type IAT (when it is in the unprocessed state as proIAT), the 40-kDa band may contain a mixture of both proteins. These results suggest that the unprocessed IAT^{C103S} exerts a regulatory role on wild-type IAT processing, probably affecting the self-processing. Therefore, transformants T16 and T20, which synthesize the IAT^{C103S} during the culture time, have a deficient self-processing of the wild-type IAT, giving rise to lower amounts of the β subunit and to a low benzylpenicillin production.

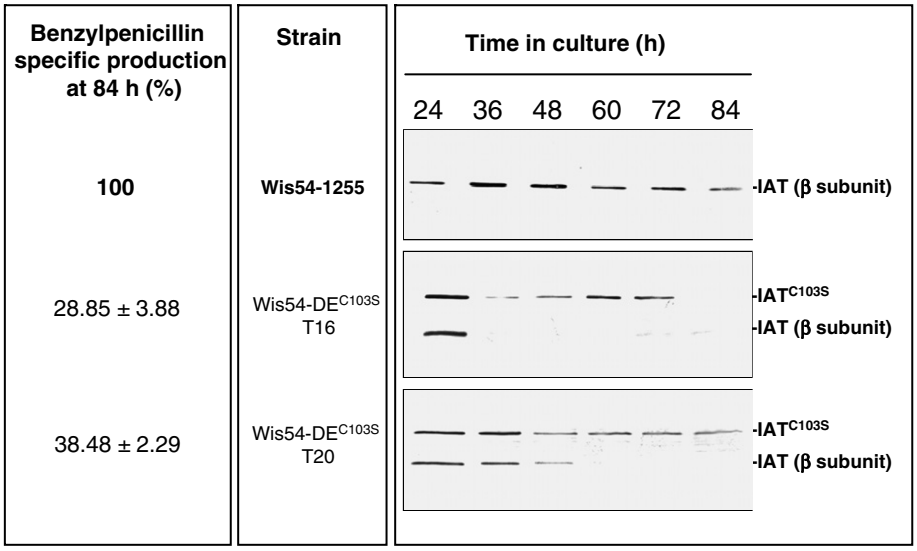

Fig. 4. Representative Western blots carried out with anti-IAT antibodies, showing the IAT and IAT^{C103S} synthesis profile in the control Wis54-1255 strain and in transformants T16 and T20 of the Wis54-*DE*^{C103S} strain. Three micrograms of protein (determined by Bradford assay) were loaded in each lane. Data regarding the relative benzylpenicillin specific production (%) of each transformant in Fig. 3C is indicated on the left column of this figure.

3.5. The unprocessed IAT^{C103S} affects the self-processing of the wild-type IAT in the heterologous host E. coli

In light of the results obtained in *P. chrysogenum*, the effect of the proIAT^{C103S} on the processing of the wild-type IAT was studied in *E. coli*. Therefore, plasmid pαβ-*DE*^{C103S}, which is able to co-express both the *penDE*^{C103S} and the *penDE* gene, was constructed as indicated in Table 1. Co-expression was performed at 26 °C for 2 h and the formation of the β (29 kDa) subunit was used as the criterion of processing. SDS–PAGE analysis (Fig. 5A) showed the presence of the three bands, which correspond to the unprocessed mutant IAT^{C103S} (40-kDa) and to the β and α subunits (29 kDa and 11 kDa, respectively), both generated after self-processing of the wild-type IAT. This result indicates that simultaneous co-expression of both genes in *E. coli* is a suitable system to study the regulation of self-processing of IAT. To test whether the IAT^{C103S} exerts a partial effect on IAT maturation (i.e. generation of slow-processing molecules), IAT self-processing was assessed in absence or presence of the IAT^{C103S} as indicated in Section 2. Western blot analyses (Fig. 5B) showed that the wild-type preprotein is processed into the β and α subunits. This processing was practically complete after 5 h at 26 °C (only traces of the 40 kDa protein remain at this time). However, when the *penDE*^{C103S} gene was co-expressed with the wild-type *penDE* gene, the 40-kDa band (corresponding to the mutant protein and the wild-type IAT that remained unprocessed) was present throughout the experiment. Only after 5 h at 26 °C, the 29-kDa band (corresponding to the β subunit formed by processing of wild-type IAT) was visible. Therefore, the unprocessed protein interferes with the *in vivo* processing of the recombinant IAT in the *E. coli* heterologous expression system. These results indicate that IAT is post-translationally regulated by its preprotein, the excess of which is able to delay the self-processing generating slow-processing molecules.

3.6. The IAT^{C103S} does not inhibit the in vitro acyltransferase activity of previously processed IAT

In order to assess whether the lower benzylpenicillin production of the strain which co-expresses both the *penDE*^{C103S} and the *penDE* genes (*P. chrysogenum* Wis54-*DE*^{C103S}) is a consequence of an inhibition of the acyltransferase activity exerted by the unprocessed IAT^{C103S}, *in vitro* experiments were performed. *penDE* and *penDE*^{C103S} genes were overexpressed in separate *E. coli* cultures at 26 °C for 3 h. Soluble fractions containing the active recombinant α–β heterodimer were mixed with soluble fractions containing the unprocessed IAT^{C103S}. After a pre-incubation period of 1 h or 5 h at 26 °C (to allow protein–protein interaction), 6-APA and phenylacetyl-CoA were added to the reaction (see Section 2). The *in vitro* acyltransferase activity (benzylpenicillin forming) of the previously processed IAT was not affected by the presence of the unprocessed IAT^{C103S} (Fig. 6). Therefore, the *in vivo* decrease in the benzylpenicillin biosynthesis observed in the Wis54-*DE*^{C103S} strain is unlikely due to an inhibitory effect exerted by the IAT^{C103S} on the acyltransferase activity of the previously processed enzyme (see Section 4).

4. Discussion

Proteolytic processing of many proteins (proenzymes) is a general mechanism of activation of enzymes that are synthesized as inactive proenzymes.

The IAT, encoded by the *penDE* gene, is self-processed forming an α–β heterodimer (Barredo et al., 1989; Montenegro et al., 1990; Veenstra et al., 1989; Tobin et al., 1990, 1993). When the self-processing ability of the IATs of *P. chrysogenum* and *A. nidulans* were compared to each other we observed that, whereas the *P. chrysogenum* enzyme is processed very efficiently, the *A. nidulans* proIAT was slowly processed. Although the processing site Gly102/Cys103 was identical in IATs of both fungi, the structure of the enzyme showed differences in about 25% of the amino acids, a fact that may explain the slow self-processing of the *A. nidulans* IAT. It seems that *P. chrysogenum* has evolved a mechanism which allows this fungus to perform a very efficient self-processing of the IAT avoiding the accumulation of proIAT, whereas this regulatory mechanism still persists in *A. nidulans* that accumulates a significant amount of unprocessed IAT (Fernández et al., 2003).

The immunolocalization of the proIAT^{C103S} inside peroxisomes clearly indicates that the proIAT is targeted to those organelles as reported for the mature enzyme (Müller et al., 1992). In addition, the proIAT may be transported into the peroxisomal matrix before processing. The high concentration of enzyme in these organelles

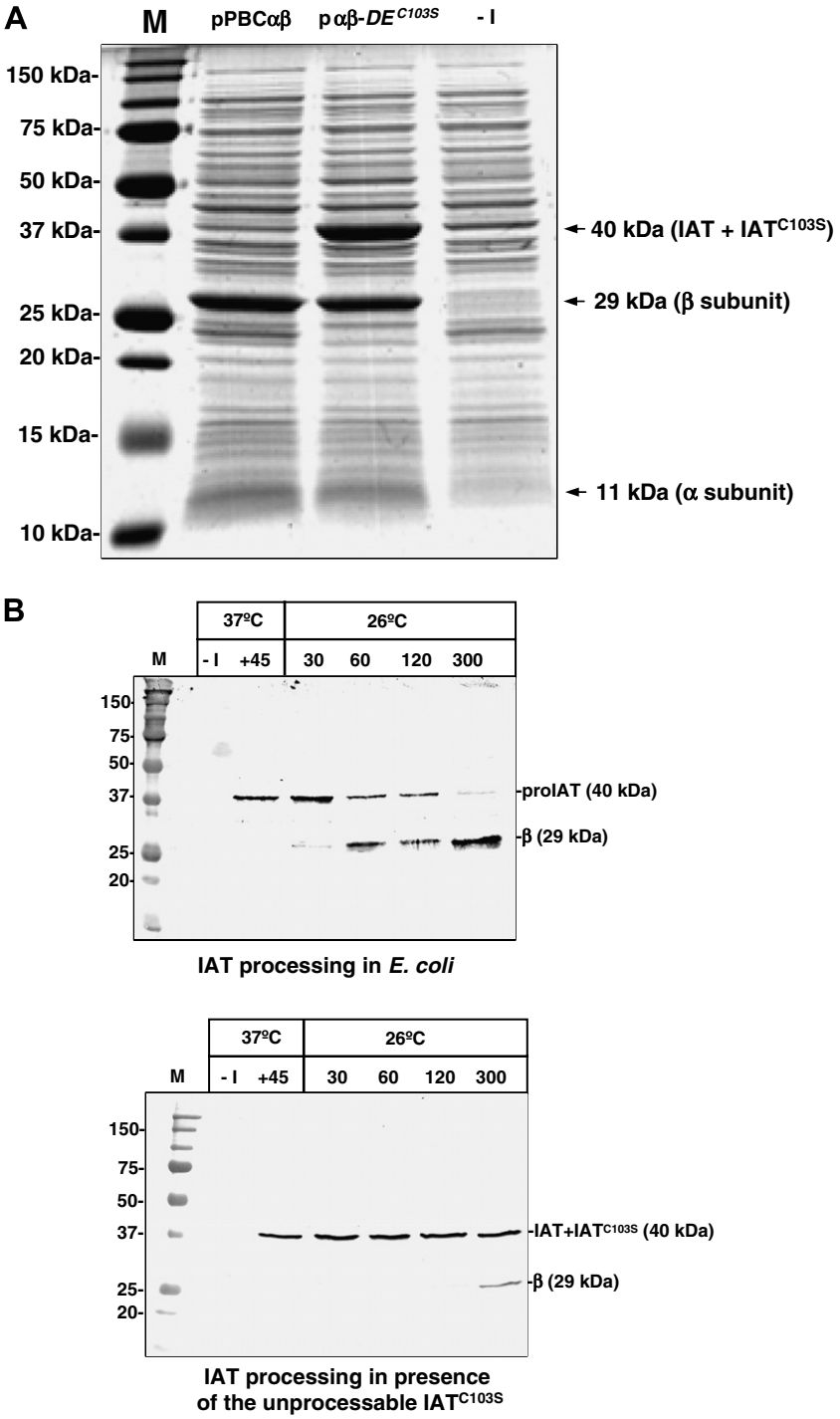

Fig. 5. Effect of the IAT^{C103S} on the processing of IAT in *E. coli*. (A) Coomassie blue stained SDS–PAGE of samples obtained from IPTG-induced (2 h at 26 °C) cells transformed with plasmid pPBCαβ (which expresses the *penDE* gene) or with plasmid pαβ-*DE*^{C103S} (expressing both the *penDE*^{C103S} and the *penDE* genes). Uninduced cells (lane -I) were used as control. Molecular weight markers (lane M) are shown on the left. (B) Western blotting (using anti-IAT antibodies) showing the processing of the recombinant IAT at 26 °C into the β subunit, either in absence (upper panel), or presence (lower panel) of the unprocessed IAT^{C103S} form. Induction of protein synthesis was achieved at 37 °C for 45 min with IPTG. Then, cells were washed to remove the IPTG and incubated at 26 °C to allow self-processing. Samples were taken after 30, 60, 120 and 300 min, diluted and analysed by Western blotting. Samples from uninduced cells are indicated as (-I). Molecular weight markers (lane M) are shown on the left in kDa.

and the adequate ambient pH value are likely to be essential for efficient self-processing.

In this work we observed that *P. chrysogenum* transformants of the npe10-*AB-C-DE*^{C103S} strain (which contains the first two genes of the penicillin pathway and the mutant *penDE*^{C103S} gene encoding the unprocessable IAT^{C103S}) lack acyltransferase activity. Therefore, they are unable to synthesize benzylpenicillin despite the proper peroxisomal location of the variant protein; i.e. the processing of proIAT *in vivo* is absolutely required for functionality of this enzyme.

The IAT appears to be a NTN (N-terminal nucleophile) enzyme (Brannigan et al., 1995) where the Cys103 is the N-terminal residue acting as nucleophile. This amino acid is essential for processing and therefore, we used the IAT^{C103S} protein variant to study the

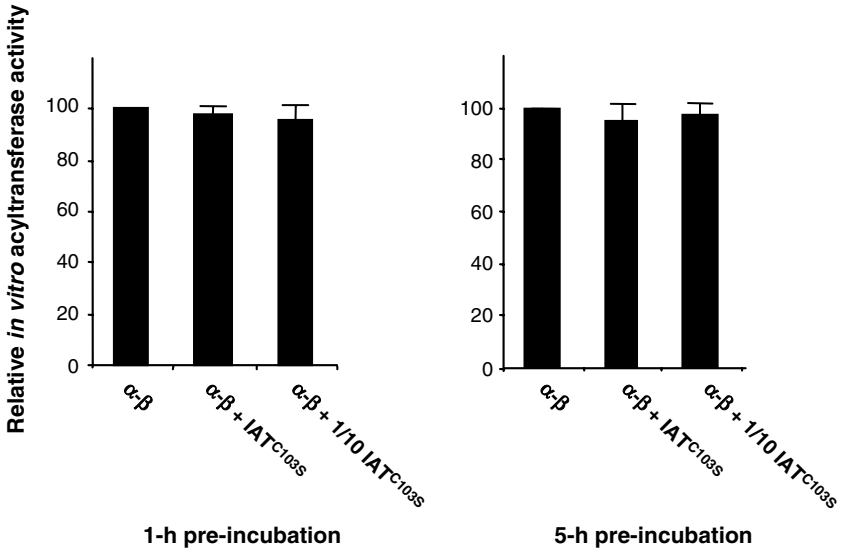

Fig. 6. Lack of inhibition of the acyltransferase activity of the mature IAT (α + β) by the unprocessed IAT^{C103S}. *In vitro* acyltransferase activity (μg of benzylpenicillin/mg protein) was assessed in absence and presence of the IAT^{C103S}. Soluble fractions containing the active recombinant IAT were mixed either with soluble extracts from uninduced *E. coli* cells (α–β), with soluble fractions containing the IAT^{C103S} (α–β + IAT^{C103S}), or with a 1/10 dilution of the latter (α–β + 1/10 IAT^{C103S}). After a pre-incubation period of 1 or 5 h at 26 °C, 6-APA and phenylacetyl-CoA were added to the reaction, which was stopped after 15 min at 26 °C with methanol. Results were expressed as the percentage of activity (%) regarding the activity provided by the recombinant IAT in absence of the IAT^{C103S}.

role of this protein (unprocessed heterodimer) in the processing activity of the wild-type IAT. We have observed that transformants of the Wis54-*DE^{C103S}* strain, which express simultaneously the wild-type *penDE* and the *penDE^{C103S}* genes, show a decrease in penicillin production with respect to the parental Wis54-1255 strain (Fig. 3). This result supports the hypothesis that the presence of the 40-kDa proIAT affects the synthesis of benzylpenicillin. We have observed that the biosynthesis of IAT^{C103S} in the Wis54-*DE^{C103S}* strain (transformants T16 and T20) leads to a reduction of the β-subunit formed by self-cleavage of normal IAT, which is only detected in the early hours of culture (Fig. 4). *In vitro* experiments using previously processed (mature) IAT (Fig. 6) proved that the acyltransferase activity of mature IAT was not affected by the presence of the preprotein. This indicates that the decrease in benzylpenicillin biosynthesis observed in transformants T16 and T20 of the Wis54-*DE^{C103S}* strain, was not simply due to an inhibitory effect of the proIAT^{C103S} on the activity of the mature α–β heterodimer. One explanation of these results is that the unprocessed 40-kDa preprotein, when accumulated, exerts a regulatory role affecting the processing of the endogenous IAT, as reported for other NTN enzymes (Seemüller et al., 1996; Böck et al., 1983; Choi et al., 1992), thus decreasing the amount of the active form. The effect of the unprocessed proIAT^{C103S} on the wild-type IAT processing was confirmed in *E. coli*, where synthesis of the variant IAT^{C103S} was easily induced (Fig. 5). The results in *E. coli* indicated that the presence of the unprocessable protein does not completely prevent the self-processing of the wild-type IAT, but caused a delay in this process generating slow-processing molecules. In the *P. chrysogenum* strain, which co-express both the *penDE* and the *penDE^{C103S}* gene, the IAT^{C103S} acts to delay the processing of the wild-type IAT, thus interfering with the generation of the α–β heterodimer. Our results suggest that the IAT forms homo-oligomers and that processing occurs in the context of those oligomers.

A similar regulatory phenomenon by the unprocessed precursor protein was observed in the 20S proteasome, another NTN enzyme (Seemüller et al., 1996). Our results support the hypothesis that processing of these enzymes may be intermolecular, i.e. one IAT molecule serving as substrate of another IAT molecule. An excess of unprocessed IAT^{C103S} could block the processing sites of the active form. A similar mechanism has been reported for the penicillin acylase from *E. coli* (Böck et al., 1983; Choi et al., 1992), a NTN enzyme that catalyzes hydrolysis of the amide bond of benzylpenicillin releasing 6-APA and phenylacetic acid (Valle et al., 1991; McVey et al., 2001; Kasche et al., 1987, 1999). The *E. coli* penicillin acylase is synthesized as an inactive precursor and cleaved initially between amino acids 263–264 releasing the β subunit (residues 264–820). The N-terminal fragment is subsequently processed into a linker region (residues 209–263) and the small N-terminal A subunit. The protein is inactive until the first cleavage, which exposes the Ser264 nucleophile (Kasche et al., 1999).

In summary, taking into account the results reported in this article, it is concluded that the *P. chrysogenum* IAT is very rapidly and efficiently self-processed into α and β subunits. Therefore, it escapes inhibition of self-processing since the preprotein is never accumulated, unlike what occurs in *A. nidulans*. Since the preprotein variant is well transported into microbodies, the lack of transport can not be the reason for the slow processing of the *A. nidulans* IAT.

Acknowledgments

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References

Aharonowitz, Y., Cohen, G., Martín, J.F., 1992. Penicillin and cephalosporin biosynthetic genes: structure, regulation, and evolution. Annu. Rev. Microbiol. 46, 461–495.

Álvarez, E., Cantoral, J.M., Barredo, J.L., Díez, B., Martín, J.F., 1987. Purification to homogeneity and characterization of the acyl-CoA: 6-APA acyltransferase of *Penicillium chrysogenum*. Antimicrob. Agents Chemother. 31, 1675–1682.

Barredo, J.L., van Solingen, P., Díez, B., Álvarez, E., Cantoral, J.M., Kattevilder, A., Smaal, E.B., Groenen, M.A.M., Veenstra, A.E., Martín, J.F., 1989. Cloning and

characterization of the acyl-coenzyme A: 6-aminopenicillanic-acid-acyltransferase gene of *Penicillium chrysogenum*. Gene 83, 291–300.

Böck, A., Wirth, R., Schmid, G., Schumacher, G., Lang, G., Buckel, P., 1983. The penicillin acylase from *Escherichia coli* ATCC11105 consists of two dissimilar subunits. FEMS Microbiol. Lett. 20, 135–139.

Brannigan, J.A., Dodson, G., Duggleby, H.J., Moody, P.C.E., Smith, J.L., Tomchick, D.R., Murzin, A.G., 1995. A protein catalytic framework with an N-terminal nucleophile is capable of self-activation. Nature 378, 416–419.

Cantoral, J.M., Díez, B., Barredo, J.L., Álvarez, E., Martín, J.F., 1987. High frequency transformation of *Penicillium chrysogenum*. Biotechnology 5, 494–497.

Casqueiro, J., Bañuelos, O., Gutiérrez, S., Hijarrubia, M.J., Martín, J.F., 1999. Intrachromosomal recombination between direct repeats in *Penicillium chrysogenum*: gene conversion and deletion events. Mol. Gen. Genet. 261, 994–1000.

Choi, K.S., Kim, J.A., Kang, H.S., 1992. Effects of site-directed mutations on processing and activities of penicillin G acylase from *Escherichia coli* ATCC11105. J. Bacteriol. 174, 6270–6276.

Díez, B., Álvarez, E., Cantoral, J.M., Barredo, J.L., Martín, J.F., 1987. Isolation and characterization of *pyrG* mutants of *Penicillium chrysogenum* by resistance to 5′-fluorotic acid. Curr. Genet. 12, 277–282.

Duggleby, H.J., Tolley, S.P., Hill, C.P., Dodson, E.J., Dodson, G., Moody, P.C., 1995. Penicillin acylase has a single-amino-acid catalytic centre. Nature 373, 264–268.

Fernández, F.J., Cardoza, R.E., Montenegro, E., Velasco, J., Gutiérrez, S., Martín, J.F., 2003. The isopenicillin *N* acyltransferases of *Aspergillus nidulans* and *Penicillium chrysogenum* differ in their ability to maintain the 40-kDa alphabeta heterodimer in an undissociated form. Eur. J. Biochem. 270, 1958–1968.

Fierro, F., Barredo, J.L., Díez, B., Gutiérrez, S., Fernández, F.J., Martín, J.F., 1995. The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. Proc. Natl. Acad. Sci. USA 92, 6200–6204.

Fierro, F., García-Estrada, C., Castillo, N.I., Rodríguez, R., Velasco-Conde, T., Martín, J.F., 2006. Transcriptional and bioinformatic analysis of the 56.8 kb DNA region amplified in tandem repeats containing the penicillin gene cluster in *Penicillium chrysogenum*. Fungal Genet. Biol. 43, 618–629.

García-Estrada, C., Vaca, I., Lamas-Maceiras, M., Martín, J.F., 2007. In vivo transport of the intermediates of the penicillin biosynthetic pathway in tailored strains of *Penicillium chrysogenum*. Appl. Microbiol. Biotechnol. 76, 169–182.

Hewitt, L., Kasche, V., Lummer, K., Lewis, R.J., Murshudov, G.N., Verma, C.S., Dodson, G.G., Wilson, K.S., 2000. Structure of a slow processing precursor penicillin acylase from *Escherichia coli* reveals the linker peptide blocking the active-site cleft. J. Mol. Biol. 302, 887–898.

Kasche, V., Haufler, U., Markowsky, D., Melnyk, S., Zeich, A., Galunsky, B., 1987. Penicillin amidase from *E. coli*. Ann. NY Acad. Sci. 501, 97–102.

Kasche, V., Lummer, K., Nurk, A., Piotraschke, E., Rieks, A., Stoeva, S., Voelter, W., 1999. Intramolecular autoproteolysis initiates the maturation of penicillin amidase from *Escherichia coli*. Biochim. Biophys. Acta 1433, 76–86.

Laich, F., Fierro, F., Martin, J.F., 2003. Production of penicillin by fungi growing on food products: identification of a complete penicillin gene cluster in *Penicillium griseofulvum* and a truncated cluster in *Penicillium verrucosum*. Appl. Environ. Microbiol. 68, 1211–1219.

Lamas-Maceiras, M., Vaca, I., Rodríguez, E., Casqueiro, J., Martín, J.F., 2006. Amplification and disruption of the phenylacetyl-CoA ligase gene of *Penicillium chrysogenum* encoding an aryl-capping enzyme that supplies phenylacetic acid to the isopenicillin *N* acyltransferase. Biochem. J. 395, 147–155.

MacCabe, A.P., van Liempt, H., Palissa, H., Unkles, S.E., Riach, M.B.R., Pfeifer, E., von Doren, H., Kinghorn, J.R., 1991. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. J. Biol. Chem. 266, 12646–12654.

Markaryan, A., Lee, J.D., Sirakova, T.D., Kolattukudy, P.E., 1996. Specific inhibition of mature fungal serine proteinases and metalloproteinases by their propeptides. J. Bacteriol. 178, 2211–2215.

Martín, J.F., Liras, P., 1989. Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. Annu. Rev. Microbiol. 43, 173–206.

Martín, J.F., Ingolia, T.D., Queener, S.W., 1990. Molecular genetics of penicillin and cephalosporin antibiotic biosynthesis. In: Leong, S.A., Berka, R. (Eds.), Molecular Industrial Mycology. Marcel Dekker, New York, USA, pp. 149–195.

McVey, C.E., Walsh, M.A., Dodson, G.G., Wilson, K.S., Brannigan, J.A., 2001. Crystal structures of penicillin acylase enzyme–substrate complexes: structural insights into the catalytic mechanism. J. Mol. Biol. 313, 139–150.

Montenegro, E., Barredo, J.L., Gutiérrez, S., Díez, B., Alvarez, E., Martín, J.F., 1990. Cloning, characterization of the acyl-CoA: 6 amino penicillanic acid acyltransferase gene of *Aspergillus nidulans* and linkage to the isopenicillin N synthase gene. Mol. Gen. Genet. 221, 322–330.

Müller, W.H., van der Krift, T.P., Krouwer, A.J., Wosten, H.A., van der Voort, L.H., Smaal, E.B., Verkleij, A.J., 1991. Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. EMBO J. 10, 489–495.

Müller, W.H., Bovenberg, R.A., Groothuis, M.H., Kattevilder, F., Smaal, E.B., Van der Voort, L.H., Verkleij, A.J., 1992. Involvement of microbodies in penicillin biosynthesis. Biochim. Biophys. Acta 1116, 210–213.

Müller, W.H., Essers, J., Humbel, B.M., Verkleij, A.J., 1995. Enrichment of *Penicillium chrysogenum* microbodies by isopycnic centrifugation in nycodenz as visualized with immuno-electron microscopy. Biochim. Biophys. Acta 1245, 215–220.

Park, C.H., Lee, S.J., Lee, S.G., Lee, W.S., Byun, S.M., 2004. Hetero- and autoprocessing of the extracellular metalloprotease (Mpr) in *Bacillus subtilis*. J. Bacteriol. 186, 6457–6464.

Seemüller, E., Lupas, A., Baumeister, W., 1996. Autocatalytic processing of the 20S proteasome. Nature 382, 468–470.

Tobin, M.B., Fleming, M.D., Skatrud, P.L., Miller, J.R., 1990. Molecular characterization of the acyl-coenzyme A: isopenicillin *N* acyltransferase gene (*penDE*) from *Penicillium chrysogenum* and *Aspergillus nidulans* and activity of recombinant enzyme in *E. coli*. J. Bacteriol. 172, 5908–5914.

Tobin, M.B., Baldwin, J.E., Cole, S.C.J., Miller, J.R., Skatrud, P.L., Sutherland, J.D., 1993. The requirement for subunit interaction in the production of *Penicillium chrysogenum* acyl-coenzyme A: isopenicillin *N* acyltransferase in *Escherichia coli*. Gene 132, 199–206.

Tobin, M.B., Cole, S.C., Miller, J.R., Baldwin, J.E., Sutherland, J.D., 1995. Amino acid substitutions in the cleavage site of acyl-coenzyme A: isopenicillin *N* acyltransferase from *Penicillium chrysogenum*: effect on proenzyme cleavage and activity. Gene 162, 29–35.

Valle, F., Balbas, P., Merino, E., Bolivar, F., 1991. The role of penicillin amidases in nature and in industry. Trends Biochem. Sci. 16, 36–40.

Veenstra, A.E., van Solingen, P., Huininga-Muurling, H., Koekman, B.P., Groenne, M.A.M., Smaal, E.B., Kattevilder, A., Alvarez, E., Barredo, J.L., Martín, J.F., 1989. Cloning of penicillin biosynthetic genes. In: Hershberger, C.L., Queener, S.W., Hegeman, G. (Eds.), Genetics and Molecular Biology of Industrial Microorganisms. American Society for Microbiology, Washington, DC, USA, pp. 262–269.

Waterham, H.R., Titorenko, V.I., Haima, P., Cregg, J.M., Harder, W., Veenhuis, M., 1994. The *Hansenula polymorpha* PER1 gene is essential for peroxisome biogenesis and encodes a peroxisomal matrix protein with both carboxy- and amino-terminal targeting signals. J. Cell Biol. 127, 737–749.

Whiteman, P.A., Abraham, E.P., Baldwin, J.E., Fleming, M.D., Schofield, C.J., Sutherland, J.D., Willis, A.C., 1990. Acyl coenzyme A: 6-aminopenicillanic acid acyltransferase from *Penicillium chrysogenum* and *Aspergillus nidulans*. FEBS Lett. 262, 342–344.