Molecular characterization of the *Acremonium chrysogenum* *cefG* gene product: the native deacetylcephalosporin C acetyltransferase is not processed into subunits

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Constructions starting at each of the three in-frame ATG codons of the *Acremonium chrysogenum* *cefG* gene (Met¹, Met⁴⁶ and Met⁶⁰) were expressed in *Escherichia coli*, obtaining proteins of 49, 44 and 43 kDa, respectively. All three proteins showed deacetylcephalosporin C (DAC) acetyltransferase activity. The native *A. chrysogenum* DAC acetyltransferase was purified to electrophoretic homogeneity by immunoaffinity chromatography. It showed a molecular mass of 50 kDa by filtration in calibrated Sephadex G-75 SF or Superose 12 (FPLC) columns. The N-terminal end of the pure DAC acetyltransferase was Met-Leu-Pro-Ser-Ala-Gln-Val-Ala-Arg-Leu, which matched perfectly the deduced amino acid sequence starting at Met¹. The putative α - and β -subunits of DAC acetyltransferase were also obtained in *E. coli* but showed no enzymic activity either

separately or in combination. Immunoblotting (Western) analysis revealed that the 50 kDa DAC acetyltransferase showed high protein levels in *A. chrysogenum* cultures at 72 and 96 h and decreased sharply thereafter, but in all cases no detectable processing of the enzyme into subunits was found. Three different *A. chrysogenum* strains (including the wild-type Brotzu strain and two high-cephalosporin-producing mutants) showed the same unprocessed 50 kDa DAC acetyltransferase. The non-producer mutant ATCC 20371 showed no DAC acetyltransferase protein band but formed a normal transcript of 1.4 kb.

Key words: cephalosporin C, immunodetection, protein processing.

INTRODUCTION

The final step in the cephalosporin C biosynthetic pathway is the conversion of deacetylcephalosporin C (DAC) into cephalosporin C by the enzyme DAC acetyltransferase, which uses acetyl CoA as donor of the acetyl group [1,2] (Figure 1). *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*) mutants that are impaired in cephalosporin C biosynthesis and accumulate DAC were reported several years ago [3,4]. Cell-free extracts of these mutants are unable to convert DAC into cephalosporin C, which is at variance with what occurs with the wild type [5]. However, it is unknown if these mutants form an inactive DAC acetyltransferase or whether they are blocked in transcription or

translation of the gene encoding acetyl-CoA:DAC O-acetyltransferase (DAC-AT).

The enzyme DAC-AT was initially observed in permeabilized cells of *A. chrysogenum* [6] and was partially purified by Scheidegger and co-workers [7], who proposed a molecular mass of 70 ± 5 kDa. Later, Matsuyama et al. [8] purified the enzyme and estimated a molecular mass of 55 kDa by gel filtration. These authors proposed that the enzyme was a heterodimer of two subunits of 27 and 14 kDa, but did not explain the subunit composition of the native enzyme since a simple heterodimer (27 + 14 kDa) would not explain the observed molecular mass of 55 kDa. The controversy about the molecular mass and the subunit structure of DAC-AT has remained open until now.

Figure 1 Reaction catalysed by the DAC-AT encoded by the *cefG* gene of *A. chrysogenum*

The reverse reaction is catalysed by a different enzyme (cephalosporin C acetylhydrolase).

Abbreviations used: DAC, deacetylcephalosporin C; DAC-AT, acetyl-CoA:DAC O-acetyltransferase; LB, Luria–Bertani.

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We cloned the *cefG* gene from *A. chrysogenum* CW19 encoding DAC-AT [9] and independently the same gene was also reported by Matsuda et al. [10] and Mathison and co-workers [11]. Each of these research groups proposed a different size for the *cefG* gene based on selection of three different ATG translation-initiation codons in the open reading frame. The *cefG* gene as proposed by Gutiérrez et al. [9] contains two introns and encodes a protein of 444 amino acids with a deduced molecular mass of 49269 Da. This gene is transcribed as an mRNA of 1.4 kb [9,12] and complements the deficiency of DAC-AT in the non-producer mutant *A. chrysogenum* ATCC 20371, restoring cephalosporin biosynthesis to this strain.

On the other hand, Matsuda and co-workers [10] proposed that the *cefG* gene started at the third in-frame ATG codon. This resulted in a protein of 385 amino acids that was assumed to be processed into a leader peptide of 12 amino acids, a subunit of 247 amino acids (27 kDa) and another of 126 amino acids (14 kDa), although they reported that the active form of the enzyme had a molecular mass of 55 kDa. Finally, Mathison and co-workers [11] proposed that the gene would start at the second in-frame ATG codon, resulting in a protein of 399 amino acids (44 kDa), and did not study whether it was processed or not.

It was, therefore, of great interest to clarify the nature of the *cefG* gene product and to establish unequivocally whether the DAC-AT protein is processed *in vivo* or not, using antibodies against this enzyme. Moreover, processing might be strain-dependent or time-dependent during the fermentation. We describe in this article a characterization of the DAC-AT, showing that in three different *A. chrysogenum* strains the DAC-AT is not processed at any time during the culture. In addition, the mutant strain ATCC 20371 does not show any trace of immunodetectable DAC-AT, although the *cefG* gene is expressed into a transcript of the adequate size.

MATERIALS AND METHODS

Strains used

Four strains of *A. chrysogenum* were used in this study, including the wild-type (Brotzu) isolate ATCC 11550, the improved cephalosporin producers CW19 (ATCC 36225) [13] and C10 (ATCC 48272), a high-cephalosporin-producing strain released by PanLabs [13,14] and the non-producer mutant ATCC 20371 that is deficient in DAC-AT [9,15]. Strains were grown in LPE medium [16] for 7 days at 28 °C for sporulation. Spores collected from three plates of LPE medium ([16], composition per litre, 1 g of glucose, 2 g of yeast extract, 1.5 g NaCl, 10 g CaCl₂, 2.5 % agar; adjusted to pH 6.7 with 1 M NaOH) were suspended in 100 ml of seed medium #1 (see [17]) in 500 ml shake flasks and incubated at 25 °C for 48 h in an orbital incubator at 250 rev./min. This seed culture (5 ml) was used to inoculate 100 ml of MDFA medium (in 500 ml flasks) that were incubated at 25 °C (in triplicate) for 6 days and from which samples were taken every 24 h.

Escherichia coli JM109 (DE3) [*recA1*, *supE44*, Δ (*lac-proAB*), *endA1*, *hsdR17* (*r_K-m_K*), *gyrA96*, *relA1*, *thi*, [*F'*, *traD36*, *proAB*, *lacI^q* Δ M15], λ (DE3)] (Promega, Madison, WI, U.S.A.), which contains integrated in its chromosome the gene encoding the RNA polymerase of phage T7 under the control of the isopropyl β -D-thiogalactoside (IPTG)-inducible *lac* promoter [18], was used for overproduction of different forms of DAC-AT in *E. coli*.

Expression of proteins in *E. coli*

A series of constructs were made to overexpress different fragments (putative subunits) of DAC-AT in the expression

Table 1 Different protein forms and enzymic activity of DAC-AT obtained by expressing in *E. coli* the corresponding DNA fragments of *cefG*

Plasmid	Recombinant DAC-AT	DAC-AT activity (pkat/mg of protein)
pULact1	49 kDa protein (Met ¹)	3.4
pULact2	44 kDa protein (Met ⁴⁶)	7.9
pULact2L	44 kDa protein (Met ⁴⁶)	6.2
pULact3	43 kDa protein (Met ⁶⁰)	4.7
pULact3L	43 kDa protein (Met ⁶⁰)	5.4
pULact2C	Subunit α , 31 kDa (Met ⁴⁶)	0
pULact3C	Subunit α , 30 kDa (Met ⁶⁰)	0
pULact4	Subunit β , 14 kDa	0
pULact2C + pULact4	Subunit α , 31 kDa (Met ⁴⁶) + subunit β , 14 kDa	0
pULact3C + pULact4	Subunit α , 30 kDa (Met ⁶⁰) + subunit β , 14 kDa	0

vector pT7.7 [19] (see later in Results, and Table 1). All DNA manipulations were performed according to standard procedures [20].

E. coli transformants with each of these constructs were selected in Luria-Bertani (LB) medium supplemented with 100 μ g/ml ampicillin. For overexpression studies, one colony of each transformant was grown in 100 ml of LB medium with 100 μ g/ml ampicillin, 2 ml of each culture were inoculated into fresh LB medium with ampicillin and grown at 37 °C for 12 h at 250 rev./min until the culture reached an attenuation (D_{600}) of 0.5. At this moment IPTG (0.5 mM) was added and the culture was incubated for 3 h more. After that, the cells were collected by centrifugation at 5000 g.

Radioactive labelling of proteins

E. coli transformants were grown in LB medium as above. After induction with 0.5 mM IPTG for 1 h, 1 ml of each culture was supplemented with 2.5 μ l of a mixture of [³⁵S]methionine/cysteine (30 μ Ci/ml) (Trans ³⁵S-label®, ICN Biomedicals Inc, Irvine, CA, U.S.A.). After incubation for 15 min in the presence of the radioactive amino acids the cells were collected by centrifugation at 5000 g.

Analysis of the proteins by SDS/PAGE and autoradiography

Formation of labelled or unlabelled proteins was observed after SDS/PAGE of cell extracts in IPTG-induced and non-induced cultures. In experiments with protein labelling the SDS/polyacrylamide gels were dried and exposed to X-ray film in a 'cassette' with amplifying screens (Kodak X-OMAT AR) at -70 °C for 48–72 h and revealed by standard autoradiography methods.

Cell extracts: isolation of inclusion bodies and protein renaturation

The collected *E. coli* cells were suspended in lysis buffer [50 mM Tris/HCl (pH 8.0)/5 mM dithiothreitol/100 mM NaCl/1 mM EDTA (pH 8.0)/1 mg/ml lysozyme] and sonicated in a Branson B-12 sonifier (Sonic Power Company, Santa Clara, CA, U.S.A.) using five pulses of 10 s separated by refrigeration intervals of 30 s in ice. After centrifugation at 15000 g the supernatant was used as crude extract.

When formation of inclusion bodies in *E. coli* occurred, the inclusion bodies were isolated by low-speed centrifugation (3000 g; Sorvall GSA rotor, 5 min), the precipitate was washed three times with washing buffer [50 mM Tris/HCl (pH 8.0)/

5 mM dithiothreitol/100 mM NaCl/10 mM EDTA (pH 8.0)/0.5 % Triton X-100] and the inclusion-body precipitate dissolved in 8 M urea.

DAC-AT activity assay

DAC-AT activity was assayed in a reaction mixture containing 1 mM DAC, 1 mM acetyl CoA, 5 mM MgSO₄, 50 mM Tris/HCl (pH 7.5) and 50 µl of protein extract (about 100 µg of protein) in a total reaction volume of 100 µl. The reaction was incubated at 30 °C for 30 min and stopped with 100 µl of methanol.

Formation of cephalosporin C was quantified by HPLC in a Beckman System Gold apparatus with a µBondapak C₁₈ Column (Waters Associates, Milford, MA, U.S.A.) of 300 mm × 4 mm. Cephalosporin C was eluted with a mixture of solvents A [10 mM sodium acetate/acetic acid (pH 4.5)] and B (100 % acetonitrile) using a linear gradient of 0–3 % solvent B from 0 to 2 min, and a constant 3 % solvent B from 2 to 15 min, with a flow of 1.3 ml/min. Cephalosporins were detected at 260 nm. Under these conditions, DAC was eluted with a retention time of 3 min, whereas cephalosporin C was eluted at 8 min. Pure samples of DAC and cephalosporin C were provided by F. Salto (Antibióticos, S. A., León, Spain).

Polyclonal antibodies against DAC-AT

Recombinant DAC-AT (Met¹) was purified to electrophoretic homogeneity and used for immunization of rabbits. First, DAC-AT inclusion bodies were purified as above. The renaturation was performed by diluting the dissolved inclusion bodies with Redox buffer (containing 10 mM glutathione monomer, 1 mM glutathione dimer, 50 mM Tris, 10 mM CaCl₂ and 10 mM MgCl₂ at pH 8.0) to a final concentration of 4 M urea and 100 µg of protein per ml, and supplementing it with polyethylene glycol (PEG) 6000 (at a final molar ratio of PEG:protein of 10). The renatured enzyme was shown to be active [21]. After renaturation of the protein, the DAC-AT was purified by preparative SDS/PAGE. The DAC-AT was eluted from several gels and used for immunization.

Preparation and purification of antibodies

Purified DAC-AT protein (100 µg in 500 µl of PBS), was emulsified with 500 µl of complete Freund's adjuvant (Difco Laboratories Inc., Detroit, MI, U.S.A.). This emulsion was used to immunize New Zealand rabbits by intradermal injection, using the protocol described by Dunbar and Schwoebel [22]. This immunization process was repeated once a month for 3 months using incomplete Freund's adjuvant. After the immunization was completed, blood serum was collected by centrifugation, and the IgG fraction was purified by ammonium sulphate precipitation and FPLC using a protein A-Sepharose column (Pharmacia Biotech. Inc., Uppsala, Sweden) as described by Harlow and Lane [23].

For the purification of anti DAC-AT antibodies, 20 ml of specific rabbit antiserum were mixed with ammonium sulphate to a 40 % saturation. The precipitated proteins were collected by centrifugation in a Sorvall SS34 rotor during 20 min at 15000 g and were resuspended in 4 ml of 100 mM Tris/HCl (pH 8.0). This extract was loaded on to a Sephadex G-25 (Pharmacia) gel-filtration column to remove the residual ammonium sulphate. The desalted extract was loaded on to a protein A-Sepharose HR 10/2 column for FPLC. The unbound proteins were eluted from the column with 100 mM Tris/HCl (pH 8.0) buffer and finally the purified antibodies were released with 100 mM glycine/HCl buffer (pH 2.5).

Preparation of an immunoaffinity column

The Affi-Gel Hz (Bio-Rad, Hercules, CA, U.S.A.) system was used for the preparation of an immunoaffinity column. Binding of the antibody to the matrix was performed according to the supplier's instructions. Purified IgG (4.28 mg) was used to react with 5 ml of the matrix and the efficiency of the reaction was about 37.4 % (1.6 mg of IgG were bound to the matrix).

RESULTS

Constructions for overexpression of the *cefG* gene and the DNA fragments encoding the putative DAC-AT subunits

The *cefG* gene of *A. chrysogenum* was cloned from a cDNA library constructed in Lambda ZAP (Stratagene, La Jolla, CA, U.S.A.) [21]. The expression plasmids pUAct1–4 (Table 1) containing the entire *cefG* gene or different fragments of *cefG* (Figure 2) were constructed by translational fusion of *cefG* fragments (as cDNA) from plasmid pCGC1 [24] into the poly-linker of the pT7.7 plasmid.

The expression vectors pUAct2L, 2C, 3L and 3C (Table 1) were constructed by ligation between DNA fragments obtained by PCR and pT7.7, using as template pCGC1 DNA and the following primers: pUAct2L, 5'-TGCTGCGGGATAGCCTC-3', 5'-AAGCTTTCACATTAATGACTGATC-3'; pUAct2C, 5'-TGCTGCGGGATAGCCTC-3', 5'-AAGCTTCTATGTGCCGTTGATTTCCTT-3'; pUAct3L, 5'-TGTCGCCTCAGATCGCC-3', 5'-AAGCTTTCACATTAATGACTGATC-3'; pUAct3C, 5'-TGTCGCCTCAGATCGCC-3', 5'-AAGCTTCTATGTGCCGTTGATTTCCTT-3'.

Expression of the *cefG* and the fragments encoding the putative subunits in *E. coli*

To elucidate which of the three putative DAC-ATs was the active form, constructions starting at each of the three ATG translation-initiation codons and also fragments corresponding to the two putative subunits were expressed in *E. coli* JM109 (DE3). Results of denaturing SDS/PAGE gels (Figure 3A) showed that under induction conditions the different expected proteins accumulated. Clones transformed with pUAct1 accumulated a protein of 49 kDa, whereas clones transformed with pUAct2 (or pUAct2L) showed a protein band of 44 kDa and clones containing pUAct3 (or pUAct3L) showed a band of 43 kDa after induction with IPTG. None of these bands was processed in *E. coli* to give the subunits proposed by Matsuda and co-workers [10].

Clones transformed with constructions pUAct2C and pUAct3C containing the DNA fragment corresponding to the putative α -subunit formed a protein of 31 kDa and transformants with pUAct4 (corresponding to the putative β -subunit) over-expressed a protein of about 14 kDa, as expected.

When the proteins being synthesized were labelled by a pulse of radioactive methionine/cysteine, the autoradiograms revealed very clearly the proteins that were synthesized from each construction (Figure 3B). They corresponded to the major proteins observed by SDS/PAGE.

Expression of the DAC-AT proteins in soluble form

To determine the enzymic activity of the different DAC-AT forms or of mixtures of the putative subunits, a soluble form of these proteins was required. Under optimal expression conditions (37 °C and 0.5 mM IPTG), a large amount of the different proteins was obtained, but they accumulated as inclusion bodies that were easily purified but had no enzyme activity. Reducing

Figure 2 Constructs obtained from the *A. chrysogenum cefG* gene (as cDNA) to overexpress the corresponding polypeptides in *E. coli* from the T7 promoter (thick arrow)

Constructs pULact1–4 were obtained as translational fusions of *cefG* fragments using the indicated restriction sites. Constructs pULact2L, 2C, 3L and 3C were assembled by PCR using the oligonucleotides described in the Results section so that the corresponding proteins started at Met¹, Met⁴⁶ and Met⁶⁰.

Figure 3 Proteins formed by overexpressing in *E. coli* the constructs of Figure 2 after induction with IPTG

(A) SDS/PAGE of *E. coli* cell extracts. (B) Autoradiography of the gel in (A) showing the ³⁵S-labelled proteins when the expression was performed in presence of [³⁵S]methionine/cysteine (see Materials and methods). Lanes 1 and 6, molecular-mass standards (sizes in kDa are indicated on the left); lane 2, cells containing pULact1; lane 3, pULact2; lane 4, pULact2C; lane 5, pULact2L; lane 7, pULact3; lane 8, pULact3C; lane 9, pULact3L; lane 10, pULact4.

the temperature to 30 °C and the IPTG concentration to 0.1 mM resulted in formation of smaller amounts of proteins that did not form inclusion bodies and showed enzyme activity. Lowering the temperature to 30 °C but maintaining high IPTG concentrations was not satisfactory since inclusion bodies were still formed.

DAC-AT activity of the proteins expressed in *E. coli*

The different soluble proteins obtained in *E. coli* by growing the clones in LB medium supplemented with 0.1 mM IPTG at 30 °C for 18 h were assayed for DAC-AT activity. All cell extracts were pre-incubated with 1 mM clavulanic acid to inactivate the β -

lactamases formed from the ampicillin-resistance gene present in the expression plasmid to avoid interference with the DAC-AT activity assays.

As shown in Table 1, expression of the three constructs starting at Met¹, Met⁴⁶ (two different constructs, pULact2 and pULact2L) and Met⁶⁰ (two constructs, pULact3 and pULact3L) resulted in proteins with DAC-AT activity. These results indicated that the N-terminal region of DAC-AT up to Met⁶⁰ is dispensable for DAC-AT activity.

Interestingly, the proteins corresponding to the putative subunits, separately or in combinations of α - and β -subunits, showed no DAC-AT activity. The products of two different constructs

Figure 4 Purification to electrophoretic homogeneity of the DAC-AT from *A. chrysogenum* C10 by immunoaffinity chromatography

SDS/PAGE of: lane 1, crude extract; lane 2, washing eluate of the column with 0.15 M NaCl; lane 3, molecular-mass markers (sizes in kDa are indicated on the left); lane 4, washing eluate with 0.5 M NaCl; lanes 5 and 6, DAC-AT eluted from the column with 100 mM Tris/glycine (pH 2.5). The DAC-AT protein band is indicated with an arrow on the right.

with the putative α -subunit starting either at Met⁴⁶ or at Met⁶⁰ were combined with subunit β and in both cases there was no DAC-AT activity.

These results suggested that the mixture of the two subunits is not enzymically active but did not provide information about the structure of the active form of the DAC-AT. Therefore, purification of the native form of the DAC-AT from *A. chrysogenum* C10 was performed.

Immunoaffinity chromatography purification of the DAC-AT

The DAC-AT was purified to electrophoretic homogeneity in a single step by immunoaffinity using the Affi-Gel Hz System, with immobilized anti-DAC-AT antibodies prepared as indicated in Materials and methods. The enzyme was eluted with 100 mM glycine/HCl buffer (pH 2.5; Figure 4). The single protein band eluted from the column was enzymically active and gave a clear reaction with antibodies against DAC-AT. The activity of the purified DAC-AT after the final purification step was 15.25 pkat (pmol of cephalosporin C formed per s) per mg of protein, similar to the 0.13 units (21.6 pkat) reported by Matsuyama et al. [8].

The molecular mass of the native DAC-AT is 50 kDa

The purified DAC-AT obtained by immunoaffinity chromatography showed in SDS/PAGE an approximate molecular mass of 49 kDa by comparison with molecular-mass standards in the same gel (Figure 4). There was no evidence of the separation of the purified protein in two different subunits in the SDS/PAGE gel.

To determine precisely the molecular mass of the native protein, a protein extract was filtered through two different columns; Sephadex G-75 SF and the FPLC column Superose 12 30/10 calibrated with ribonuclease A (13.7 kDa), chymotrypsinogen (30 kDa), ovalbumin (43 kDa) and BSA (67 kDa). The dead volume of the columns was established with Dextran Blue (2000 kDa). The DAC-AT molecular mass established by Sephadex G-75 was 50 ± 1 kDa (K_{av} 0.1530) and the molecular mass determined by Superose 12 FPLC filtration was 52 ± 1 kDa (K_{av} 0.3542).

Figure 5 Immunoblotting (Western) analysis of the DAC-AT in cultures of *A. chrysogenum* C10 at 24, 48, 72, 96, 120 and 144 h of incubation

Note that the levels of DAC-AT (arrow) are very low at 24 and 48 h and increase substantially at 72 and 96 h decreasing thereafter (arrow). Anti-DAC-AT antibodies were obtained as indicated in Materials and methods. The sizes (in kDa) of the molecular-mass markers are shown by arrows on the left. Note that there are no DAC-AT bands of molecular mass lower than 50 kDa corresponding to the putative subunits.

These results indicate that the native enzyme of *A. chrysogenum* C10 has a molecular mass of 50–52 kDa that corresponds to the mass of the protein starting at the first ATG codon (Met¹).

Sequence of the N-terminal end of the purified DAC-AT

The N-terminal sequence of the DAC-AT protein obtained by immunoaffinity purification was Met-Leu-Pro-Ser-Ala-Gln-Val-Ala-Arg-Leu, which fully agrees with the sequence deduced from the gene starting at the first ATG codon (Met¹).

DAC-AT in cells at different times in the cultures: the 50 kDa DAC-AT is not processed at late times

Since the formation of the putative α - and β -subunits might occur late during the cephalosporin fermentations, immunoblotting studies were performed using extracts of samples taken at different times throughout the fermentation. As shown in Figure 5, DAC-AT activity started to be detected at 48 h following the rapid growth phase and showed a peak in cells at 72 and 96 h, coinciding with the phase of antibiotic production, and decreased after 120 h when the rate of cephalosporin synthesis declined.

The anti-DAC-AT antibodies showed activity against the α - and β -subunits expressed in *E. coli* and should react with the subunits of the *A. chrysogenum* DAC-AT if they were present. In all cases the DAC-AT protein detected was of about 50 kDa, as shown in Figure 5, and there were no traces of processed subunits.

Different strains with increased cephalosporin production contained a similar DAC-AT

The lack of subunits in the *A. chrysogenum* C10 DAC-AT does not exclude the possibility that they may occur in other *A. chrysogenum* strains. We tested four available strains, including the wild-type isolate *A. chrysogenum* ATCC 11550 (Brotzu strain) and the improved cephalosporin producers CW19 and C10 in addition to the DAC-AT-deficient mutant *A. chrysogenum* ATCC 20371. There was a clear increase in the levels of DAC-AT protein from the wild-type *A. chrysogenum* to the high-producer *A. chrysogenum* C10, as expected, in samples taken at 96 h of

Figure 6 (A) Immunoblotting analysis of DAC-AT in four different strains of *A. chrysogenum*, and (B) transcripts of the *cefG* and β -actin genes

(A) Lane 1, wild-type (Brotzu) strain; lane 2, *A. chrysogenum* CW19; lane 3, *A. chrysogenum* C10; lane 4, *A. chrysogenum* ATCC 20371. Extracts were obtained from cells at 96 h. Note the absence of processed forms in all strains. (B) Transcripts of the *cefG* and β -actin genes in the wild-type (Brotzu) strain (lanes 1 and 3) and *A. chrysogenum* ATCC 20371 (lanes 2 and 4). Lanes 1 and 2 show the *cefG* mRNA and lanes 3 and 4 the β -actin transcript as an internal control for the amount of RNA in the same preparations as in lanes 1 and 2.

fermentation (Figure 6A). However, in all cases the mobility of the band showed a protein of the same molecular mass (50 kDa), suggesting that there is no processing of DAC-AT in any of these strains.

A. chrysogenum mutant ATCC 20371 shows a normal 1.4 kb transcript but does not show DAC-AT protein

As shown in Figure 6(A), the non-producer strain ATCC 20371 lacks immunodetectable DAC-AT, thus explaining the inability of this strain to produce cephalosporin. However this mutant showed a normal *cefG* transcript of 1.4 kb of similar intensity in Northern-blot hybridizations as the wild-type (Brotzu) strain (Figure 6B). It seems, therefore, that this strain contains a mutation that prevents translation or results in a premature termination of the formation of the DAC-AT polypeptide.

DISCUSSION

The DAC-AT of *A. chrysogenum* was initially estimated to have a molecular mass of about 70 kDa [7]. However, later Matsuyama et al. [8] purified the enzyme and reported a molecular mass of 55 kDa. This size correlates well with the molecular mass of 49269 Da deduced from the gene cloned in our laboratory [9]. As shown in this article, purification of the DAC-AT by immunoaffinity chromatography resulted in a single protein band and showed a molecular mass of 49 kDa in SDS/PAGE,

50 kDa by Sephadex G-75 SF gel filtration and 52 kDa by Superose 12 FPLC gel filtration.

Matsuyama and co-workers [8] purified the enzyme (55 kDa) and reported that it was highly unstable. They described the formation of two subunits of molecular mass 27 and 14 kDa, but the fate of the remaining polypeptide (8 kDa according to the deduced size of the cloned gene) was not explained. Formation of the putative subunits was proposed to occur by proteolytic processing. We could not detect formation of the subunits *in vivo* by immunodetection studies, using three different strains of *A. chrysogenum*. However, Matsuyama and co-workers used an industrial strain, *A. chrysogenum* IS-5, of the Asahi Chem. Co. and we cannot exclude the possibility that this particular strain has the ability to process DAC-AT.

Self-processing is known to occur in the penicillin-biosynthetic enzyme isopenicillin N:phenylacetyl-CoA acyltransferase [25] and Fernández, F. J., Montenegro, E., Velasco, J., Gutiérrez, S., Fierro, F. and Martín, J. F., unpublished work) but in this case the penicillin-biosynthetic enzyme is targeted to microbodies, whereas the DAC-AT is cytosolic and the deduced protein lacks any of the known targeting sequences [26].

The DAC-AT (50 kDa) that we observed by immunoblotting in all tested *A. chrysogenum* strains was not dissociated into subunits by boiling in SDS containing buffer before performing the electrophoresis (Figure 4), thus excluding the possibility that the DAC-AT is a heterodimer. Our results are in agreement with the molecular mass reported for the purified enzyme by Matsuyama and co-workers [8] (55 kDa) but differ in the absence of processing into subunits. It would be of interest to see if the industrial *A. chrysogenum* IS-5 strain has acquired a proteolytic activity that is absent in the three strains tested in this work.

A. chrysogenum cultures showed an immunodetectable band of DAC-AT until 120 h, although with lower intensity than at 72 and 96 h coinciding with the phase of rapid antibiotic synthesis. The immunodetected band showed a size of 50 kDa throughout the fermentation and there was no trace of specific proteolytic cleavage even at 120 h.

The immunoblotting studies have shown conclusively that the *A. chrysogenum* ATCC 20371 mutant lacks DAC-AT. This strain, isolated by Fujisawa and co-workers [15] as a cephalosporin negative mutant that accumulates DAC, shows, however, a normal transcript of 1.4 kb and, therefore, the mutation probably results in a truncated protein or in the inability to translate the mRNA.

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