Mol Gen Genet (1991) 225:56–64
002689259100008J

MGG


# Expression of the *pen*DE gene of *Penicillium chrysogenum* encoding isopenicillin N acyltransferase in *Cephalosporium acremonium*: production of benzylpenicillin by the transformants

**Santiago Gutiérrez, Bruno Díez, Emilio Alvarez, Jose L. Barredo and Juan F. Martín**

Section of Microbiology, Department of Ecology, Genetics and Microbiology, Faculty of Biology, University of León, E-24071 León, Spain

Received July 4, 1990

**Summary.** No DNA sequence homologous to the *pen*DE gene of *Penicillium chrysogenum* was found in the genome of three different strains of *Cephalosporium acremonium*. The *pcb*C-*pen*DE gene cluster of *P. chrysogenum* complemented the isopenicillin N synthase deficiency of *C. acremonium* mutant N2 and resulted in the production of penicillin, in addition to cephalosporin, in cultures supplemented with phenylacetic acid. The penicillin formed was identified as benzylpenicillin by HPLC and NMR studies. The *pen*DE gene of *P. chrysogenum* is expressed in *C. acremonium* forming a transcript of 1.15 kb. The transcript is processed and translated in *C. acremonium* resulting in the formation of acyl CoA: isopenicillin N acyl transferase. When the *pen*DE gene was introduced into a cephalosporin producing strain, the total titre of β-lactam antibiotics comprised distinct proportions of penicillin and cephalosporin in different transformants. Analysis of the hybridization patterns of the DNA of *C. acremonium* transformed with the *pcb*C or *pen*DE genes indicated that integration occurs by non-homologous recombination.

**Key words:** Penicillin biosynthesis – Isopenicillin N acyltransferase – *pen*DE gene – Expression in *Cephalosporium acremonium* – Production of benzylpenicillin

## Introduction

The biosynthetic pathways of penicillin in *Penicillium chrysogenum* and cephalosporin in *Cephalosporium acremonium* have the first two steps in common (Martín and Liras 1989) (Fig. 1). Both antibiotics are formed by condensation of the three precursor amino acids (L-α-aminoadipic acid, L-cysteine and L-valine) to form the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine tripeptide (ACV). This tripeptide is cyclized by isopenicillin N synthase (IPNS) to form isopenicillin N (IPN), a penicillin having an α-aminoadipyl side chain. From IPN the pathway diverges in penicillin and cephalosporin producers. In *P. chrysogenum*, the α-aminoadipyl side chain of IPN is exchanged for aromatic acids such as phenylacetic or phenoxyacetic acid by the isopenicillin N acyltransferase (previously named 6-aminopenicillanic acid acyltransferase) (AAT) (Alvarez et al. 1987). This reaction does not occur in *C. acremonium* as it lacks AAT. In cephalosporin producers (but not in *P. chrysogenum*), IPN is isomerized to penicillin N by the isopenicillin N epimerase. Penicillin N is converted to deacetoxycephalosporin C by the deacetoxycephalosporin C synthetase (the so-called ring-expanding enzyme or expandase). Deacetoxycephalosporin C is hydroxylated to deacetylcephalosporin C by an α-ketoglutarate-linked dioxygenase. Acetylation of deacetylcephalosporin C to cephalosporin C is the terminal reaction of the pathway in cephalosporin-producing fungi (Martín and Liras 1989).

The three genes (*pcb*AB, *pcb*C and *pen*DE) involved in penicillin biosynthesis have now been cloned and characterized. Gene designations have been made according to the comprehensive proposal of Martín et al. (1990). The *pcb*C gene (encoding isopenicillin N synthase; previously named *ips*) has been cloned from two different strains of *P. chrysogenum* (Carr et al. 1986; Barredo et al. 1989a). The isopenicillin N acyltransferase gene (*pen*DE; previously named *aat*) has been cloned from the genome of *P. chrysogenum* (Barredo et al. 1989b) and also from *Aspergillus nidulans* (Montenegro et al. 1990). The gene encoding the tripeptide synthetase (*pcb*AB) has recently been cloned in our laboratory (Díez et al. 1990). The three genes, *pcb*AB, *pcb*C and *pen*DE, are linked together in a region of the chromosome of *P. chrysogenum* (Díez et al. 1989, 1990; Smith et al. 1990) and also of *A. nidulans* (Montenegro et al. 1990; MacCabe et al. 1990), which is amplified in high penicillin-producing strains (Barredo et al. 1989c). Similarly, two of the genes involved in the biosynthesis of cephalosporin have been characterized: *pcb*C which codes for IPNS (Samson et al. 1985) and the gene (*cef*EF) encoding the bifunctional deacetoxycephalosporin C synthe-

*Offprint requests to:* J.F. Martín

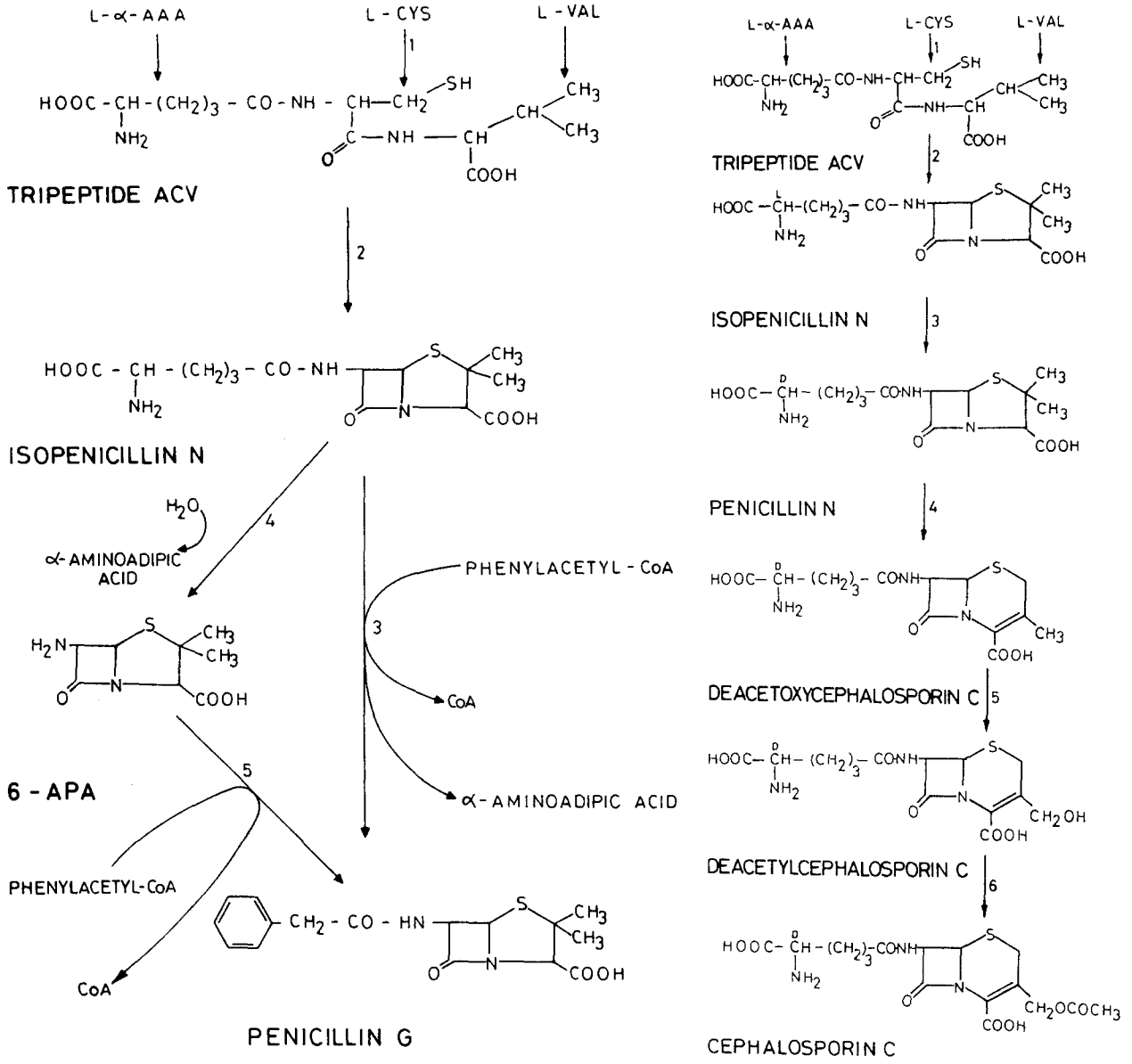


**Fig. 1.** *Left:* Biosynthetic pathway of penicillin G from the amino acids L-α-aminoadipic (AAA), L-cysteine and L-valine. 1, ACV synthetase; 2, isopenicillin N synthase; 3, isopenicillin N acyltransferase; 4, isopenicillin N amidase (6-APA forming); 5, 6-APA acyltransferase. *Right:* Biosynthetic pathway of cephalosporin C from the same component amino acids. 1, ACV synthetase; 2, isopenicillin N synthase; 3, isopenicillin N epimerase; 4, deacetoxycephalosporin C synthase; 5, deacetoxycephalosporin C hydroxylase; 6, deacetylcephalosporin C acetyltransferase. Note that the two initial steps are identical in both biosynthetic pathways

tase/deacetylcephalosporin C synthetase (Samson et al. 1987).

Protoplast transformation systems for introduction of DNA into *P. chrysogenum* and *C. acremonium* have been reported (Kolar et al. 1988; Queener et al. 1985; Cantoral et al. 1987; Díez et al. 1987; see also review by Fincham 1989). Using these transformation systems it is possible to transfer genes from penicillin-producing fungi to cephalosporin producers and vice versa. If these genes are expressed this 'directed recombination' may result in the synthesis of hybrid antibiotics (Hopwood et al. 1985) or in the production of a known β-lactam antibiotic by a different strain which might have better fermentation characteristics. Since *C. acremonium* does not have isopenicillin N acyltransferase activity, it was of great interest to transform this microorganism with the *pen*DE gene of *P. chrysogenum* and to study its expression. We present in this paper evidence showing that the *pen*DE gene (which contains three introns) is expressed in *C. acremonium* forming a 1.15 kb transcript and a functional isopenicillin N acyltransferase. This results in the production of benzylpenicillin by *C. acremonium*, a microorganism which does not produce penicillin in nature. The transforming gene is integrated into high-molecular weight DNA, presumably in the chromosome.

## Materials and methods

*Strains and culture conditions.* Three strains of *C. acremonium* were used in this work. *C. acremonium* CW-19

is a standard laboratory cephalosporin-producing strain (Zanca and Martín 1983; Pérez-Martínez and Peberdy 1985). Strain N2 is a mutant blocked in cephalosporin biosynthesis (Shirafuji et al. 1979) which accumulates ACV and is deficient in isopenicillin N synthase (Ramos et al. 1986) due to a mutation within the *pcb*C structural gene (Ramsden et al. 1989). Strain C-10 is a high cephalosporin-producing mutant (Ramos et al. 1989) of Panlabs Inc. and was kindly provided by A.L. Demain. Liquid cultures were maintained as described by Zanca and Martín (1983) using the complex medium of Caltrider and Niss (1966) supplemented with 0.8 mg/ml of potassium phenylacetate. DL-Methionine (3 mg/ml) was added at 36 h of incubation to stimulate cephalosporin biosynthesis (Caltrider and Niss 1966). The *pen*DE gene used was cloned from *P. chrysogenum* AS-P-78, a raised-titre strain (Barredo et al. 1989b) provided by Antibióticos, S.A., León, Spain. Isopenicillin N acyltransferase activity was determined as reported previously (Alvarez et al. 1987).

*Determination of benzylpenicillin and cephalosporin C.* Benzylpenicillin was quantified by bioassay using *Micrococcus luteus* ATCC 9341 as test strain; this strain is very sensitive to benzylpenicillin (minimum inhibitory concentration below 0.02 μg/ml) and insensitive to cephalosporin C up to levels of 10 mg/ml, under our experimental conditions. The titre of cephalosporin C was determined after addition of *Bacillus cereus* penicillinase (Difco, penicillin-specific) to remove penicillins from the broth followed by bioassay using *Escherichia coli* ESS 2231 (a β-lactam supersensitive strain) as test organism.

*Transformation of* C. acremonium. Preparation of *C. acremonium* protoplasts and transformation with plasmids carrying the phleomycin resistance marker (or co-transformation with EMBL3 16A/pULJL43 DNA) was carried out esentially as described previously (Cantoral et al. 1987; Díez et al. 1987), except that protoplasts were plated in TSA medium (Oxoid Ltd, Basingstoke, UK) containing 10.3% sucrose and transformants were selected by resistance to 10 μg/ml of phleomycin. A volume of 100 μl of protoplast suspension ($4 \times 10^8$ protoplasts/ml) was mixed with polyethylene glycol (PEG-6000) at a final concentration of 18% and plasmid DNA (4.5–10 μg).

*DNA isolation, Southern blotting and hybridization.* All DNA work, including plasmid isolation and purification, cleavage with restriction enzymes, Southern blotting and hybridization with probes carrying either the *pcb*C or the *pen*DE gene, was done as described previously (Barredo et al. 1989a, b; Díez et al. 1989).

*RNA isolation and Northern analysis.* Total RNA from *C. acremonium* transformants was prepared as described by Ausubel et al. (1987). RNAs were separated by electrophoresis in denaturing agarose gels as described previously (Barredo et al. 1989b; Díez et al. 1989), blotted to nitrocellulose paper and hybridized with a 1430 bp *Xmn*I-*Xba*I probe, which carries the complete *pen*DE gene of *P. chrysogenum* labelled by nick translation (Barredo et al. 1989b). The hybridization was carried out at 42° C for 20 h, and washed once with 2 × SSC, 0.1% SDS at room temperature for 15 min, once with 2 × SSC, 0.1% SDS at 42° C for 15 min and once more with 0.1 × SSC, 0.1% SDS at 65° C for 15 min (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate). The filter was exposed to X-ray film (Amersham) for 48 h.

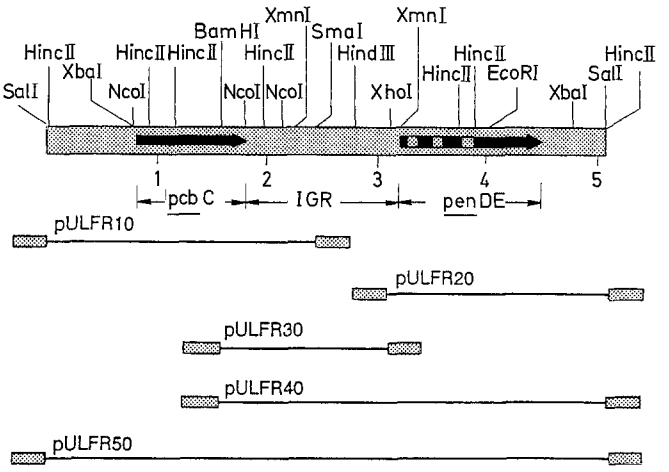


**Fig. 2.** Fragments of the *pcb*C-*pen*DE cluster of *Penicillium chrysogenum* AS-P-78 (*upper stippled* segment) subcloned in vectors pULJL43 or pULFC1 (see text for details) to obtain pULFR10, pULFR20, pULFR30, pULFR40 and pULFR50. The *pcb*C and *pen*DE genes are indicated by *thick arrows*. IGR, intergenic region. *Numbers* indicate DNA size in kb

## Results

### *Subcloning of the* pcb*C and* pen*DE genes of* P. chrysogenum *in the vector pULJL43*

Vector pULJL43 (S. Gutiérrez and J.L. Barredo, unpublished) carries the phleomycin resistance marker and contains two *Eco*RV sites. A 258 bp *Eco*RV fragment was removed to obtain a single *Eco*RV site which is useful for cloning fragments that have blunt ends. The new plasmid was named pULFC1 (4.0 kb). Using these vectors, different fragments from the previously cloned *pcb*C-*pen*DE region of *P. chrysogenum* (Barredo et al. 1989a, b; Díez et al. 1989) were subcloned forming five new plasmids pULFR10, pULFR20, pULFR30, pULFR40 and pULFR50 (Fig. 2).

pULFR10 was obtained by subcloning a 2.2 kb *Sma*I fragment isolated from pULJL7 (Barredo et al. 1989a) which carries the *pcb*C gene into *Eco*RV-digested pULFC1. pULFR20 was formed by subcloning a 2.0 kb *Xho*I-*Sal*I fragment from pULJL33, which carries the *pen*DE gene (Barredo et al. 1989b), into *Xho*I-*Sal*I-digested pULJL43. pULFR30 was constructed by subcloning a 1.3 kb *Bam*HI-*Xho*I fragment derived from pULJL33, which carries the intergenic region (IGR) between the *pcb*C and *pen*DE genes of *P. chrysogenum*, filled in with Klenow DNA polymerase, into *Eco*RV-

**Table 1.** Transformation of *Cephalosporium acremonium* N2 with plasmids carrying the *pcb*C and/or *pen*DE genes and complementation of the mutation in the cephalosporin biosynthetic pathway

| Vectors | Penicillin biosynthetic genes | DNA (μg) | Total number of transformants | Transformants assayed for antibiotic production | Transformants with positive complementation of antibiotic biosynthesis | Designation of the antibiotic-producing transformants |
|---|---|---|---|---|---|---|
| None[a] | – | 0 | 0 | 0 | 0 | – |
| pULJL43 | None | 5 | 144 | 50 | 0 | – |
| pULFR50 | *pcb*C, *pen*DE | 4.5 | 87 | 75 | 20 | TN2.1–TN2.19 |
| pULFR10 | *pcb*C | 5 | 97 | 78 | 7 | TN2.70–TN2.76 |
| EMBL3-16A/ pULJL43[b] | *pcb*C, *pen*DE | 10/1.7 | 68 | 22 | 5 | TN2.50–TN2.54 |

[a] Control transformation reactions without addition of DNA
[b] DNA from phage 16A, a derivative of EMBL3 which carries the genes *pcb*C, *pen*DE and the intergenic region between them cotransformed with pULJL43

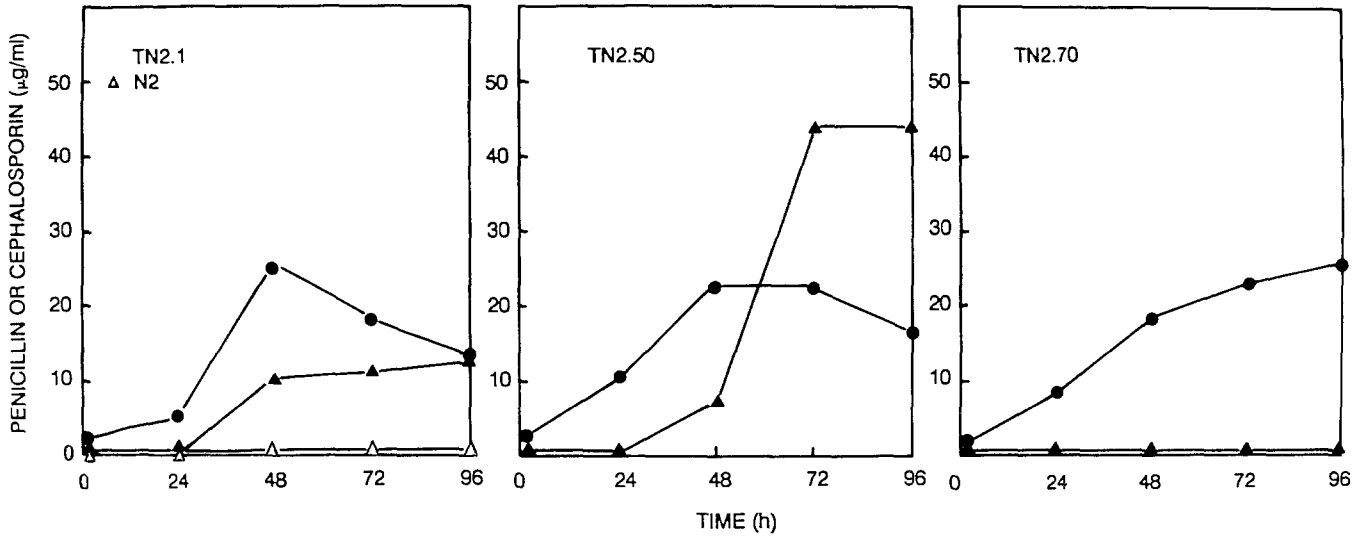


**Fig. 3.** Production of benzylpenicillin (▲—▲) and cephalosporin (●—●) C by clones of *Cephalosporium acremonium* N2 transformed with the *pcb*C gene (TN2.70) or with the *pcb*C and *pen*DE genes (TN2.50 and TN2.1) genes of *P. chrysogenum* AS-P-78. See Table 1 for details. Benzylpenicillin and cephalosporin C were quantified as described in the Materials and methods

digested pULFC1. pULFR40 was obtained by subcloning a 3.5 kb *Bam*HI fragment, which carried the *pen*DE gene, into *Bam*HI-digested pULJL43. pULFR50 contains the 5.1 kb *Sal*I fragment, which carries *pcb*C, *pen*DE and the intergenic region (Díez et al. 1989), subcloned into *Sal*I-digested pULJL43.

### *Transformation and complementation of* C. acremonium *N2 with the* pcb*C gene of* P. chrysogenum

*C. acremonium* N2 has a mutation in the isopenicillin N synthase structural gene (Ramos et al. 1986; Ramsden et al. 1989) and therefore accumulates ACV and is unable to produce cephalosporin (Shirafuji et al. 1979).

Protoplasts of N2 transformed as indicated in Materials and methods were selected by resistance to 10 μg/ml of phleomycin. The phleomycin-resistant transformants were grown in plates of Caltrider and Niss complex medium (Caltrider and Niss 1966) with 2% agar, which supported good antibiotic production. Agar plugs of each transformant were assayed after 5 days of growth at 27° C against the β-lactam supersensitive *E. coli* strain ESS-2231. Results (Table 1) showed that between 7% and 20% of the phleomycin-resistant transformants (with pULFR10 or pULFR50, respectively) were able to synthesize a β-lactam antibiotic. None of the 50 clones transformed with the vector without insert (pULJL43) were able to produce antibiotic. The untransformed N2 mutant did not show reversion to antibiotic production.

The clones obtained from each transformation which gave the largest inhibition zone in solid Caltrider and Niss medium were studied in detail in liquid cultures to quantify the concentration of antibiotic produced and to identify the antibiotic being formed. Results (Fig. 3) indicated that three different transformants (all carrying the *pcb*C gene of *P. chrysogenum*) were able to synthesize cephalosporin, in contrast to the untransformed N2 strain, which did not form any antibiotic. Transformants TN2.1 and TN2.50 which were transformed with plas-

mid pULFR50 or phage EMBL3-16A carrying the *pcb*C and *pen*DE genes, formed penicillin in addition to cephalosporin C, but TN2.70, which lacked the acyltransferase (*pen*DE) gene, was unable to form penicillin. These results suggest that the formation of penicillin in transformed clones of *C. acremonium* N2 was due to the expression of the *pen*DE gene.

*Extraction and characterization of the penicillin produced by the transformant TN2.1*

To confirm that the antibiotic produced by the transformants was penicillin, 6 ml of culture broth of transformant TN2.1 and also of *C. acremonium* strains CW-19 and $N_2$ were adjusted to pH 2.0 with phosphoric acid and the penicillin (acid form) was extracted with 4 ml of diethylether with agitation in a Vortex mixer. After removal of the aqueous phase, the penicillin in the organic phase (potassium salt) was extracted with 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.0. A solvent-soluble, penicillinase-sensitive antibiotic was extracted from cultures of transformant TN2.1 which was clearly more active against *M. luteus* than against *E. coli* (i.e. showed a penicillin-type antimicrobial action). No antibiotic could be extracted from cultures of *C. acremonium* strains $N_2$ and CW-19 using this procedure.

To identify the extracted antibiotic, 50 µl aliquots of the aqueous phase were injected into a Nucleosil C18 HPLC column (Scharlau, Barcelona, Spain) and eluted with 50 mM potassium monophosphate-methanol (70:30) at a flow rate of 1.5 ml/min. Fractions were collected and assayed for antibiotic activity. The retention time (14.6 min) and the antibiotic activity against *M. luteus* and *E. coli* agreed with those of pure benzylpenicillin (sodium salt). The nature of the benzylpenicillin formed was further confirmed by NMR spectrometry using a Bruher AM360 WB spectrometer (A.M. Veenstra, personal communication).

*Acyl-coenzyme A:isopenicillin N acyltransferase activity in transformants of* C. acremonium $N_2$

The AAT activity encoded by the *pen*DE gene was determined in cell-free extracts of culture of the three transformants (TN2.1, TN2.50 and TN2.70) of *C. acremonium* $N_2$. Results (Table 2) indicated that significant AAT activity was present in strains TN2.1 and TN2.50 (which were transformed with the *pen*DE gene) but not in the untransformed *C. acremonium* $N_2$ or in strain TN2.70 (which was transformed with the *pcb*C but not with the *pen*DE gene). These results indicate that the *pen*DE gene of *P. chrysogenum* is expressed in *C. acremonium*. Since three introns are present in the *pen*DE gene of *P. chrysogenum*, these appear to be correctly processed in *C. acremonium* as well. Furthermore, these results indicate that the pre-AAT is processed in *C. acremonium* into the enzymatically active form of AAT, as occurs in *P. chrysogenum* and *A. nidulans* (see Discussion).

**Table 2.** Acyl-coenzyme A:isopenicillin N acyltransferase activity in transformants of *C. acremonium* N2

| Strains | Penicillin biosynthetic genes | Protein in cell-free extracts (mg/ml) | AAT Specific activity picokatals[a]/mg of protein |
|---|---|---|---|
| N2 | None | 10.4 | 0 |
| TN2.1 | *pcb*C, *pen*DE | 8.5 | 1.95 |
| TN2.50 | *pcb*C, *pen*DE | 12.2 | 0.26 |
| TN2.70 | *pcb*C | 11.2 | 0 |

[a] One pkatal = one picomole of benzylpenicillin formed per second

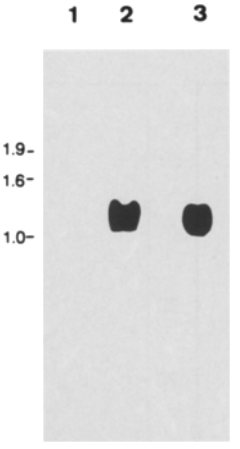


**Fig. 4.** Determination of the size of the transcript of the *pen*DE gene in *C. acremonium* TN2.1 by Northern hybridization. Total RNA from *C. acremonium* N2 (lane 1) and from the transformed TN2.1 (lane 2) was obtained as described in the Materials and methods and hybridized with a 1430 bp *Xma*I-*Xba*I probe which carries the entire *pen*DE of *P. chrysogenum* labelled with nick translation. Lane 3, for comparison of transcript sizes, is RNA of *C. acremonium* C-10 hybridized with a 1.8 kb *Sal*I-*Bam*HI fragment which carries most of the *pcb*C gene. Size markers in kb (Boehringer, RNA molecular weight marker set I) are indicated at the left

*Transcription of the* pen*DE gene in* C. acremonium *TN2.1*

Transcription of the *pen*DE gene in the *C. acremonium* transformant TN2.1 was studied by Northern hybridization. Total RNA prepared from *C. acremonium* $N_2$ and from the transformant TN2.1 was hybridized with a probe that carries the entire *pen*DE gene of *P. chrysogenum*. As observed in Fig. 4 a single transcript of about 1.15 kb in the transformant TN2.1 hybridized with the *pen*DE probe but no hybridization was observed with the RNA of the untransformed strain. The size of the transcript was identical to that of the native transcript previously identified in *P. chrysogenum* (1.15 kb) (Barredo et al. 1989b).

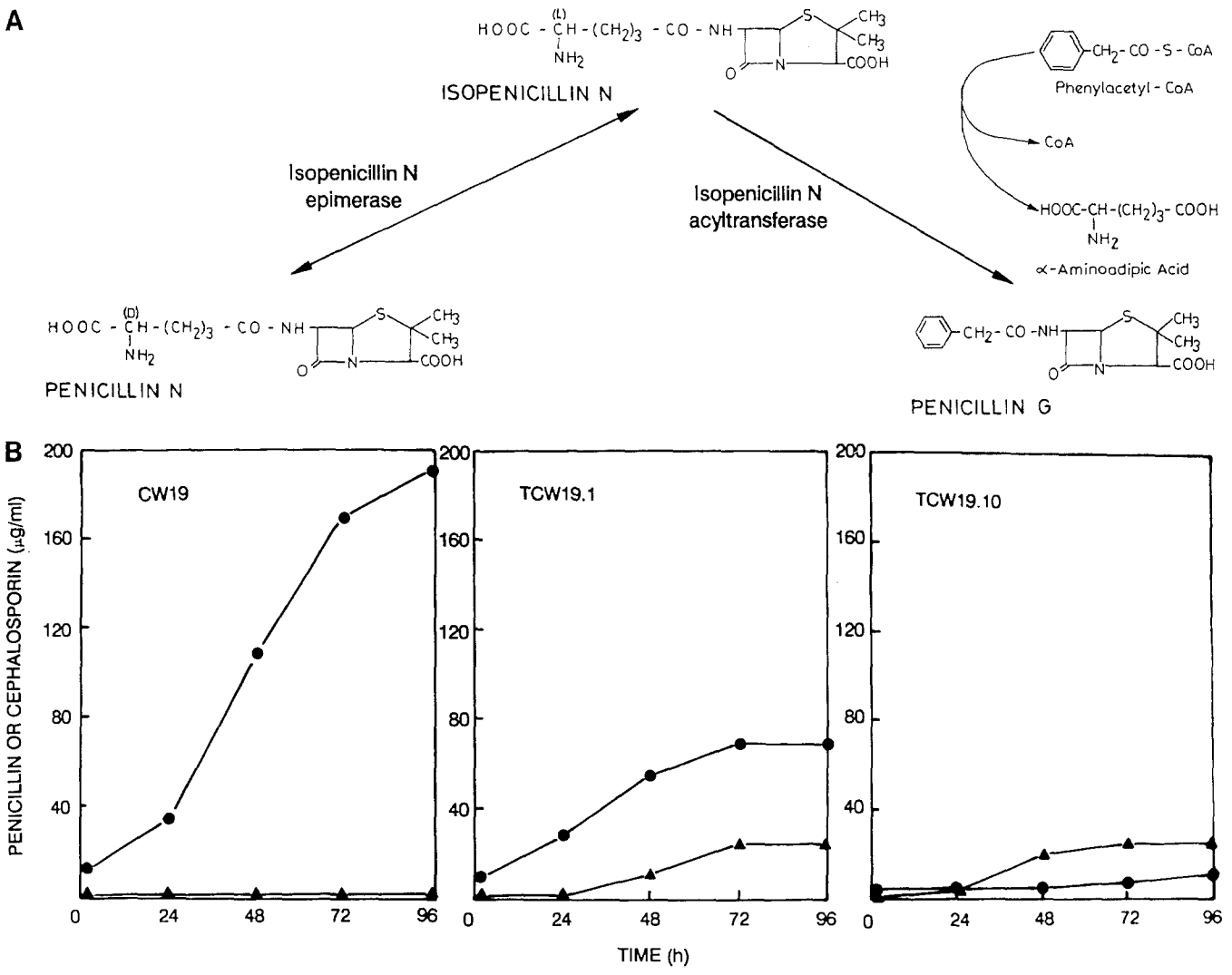


**Fig. 5.** **A** Competition between isopenicillin N epimerase and isopenicillin N acyltransferase for the intermediate isopenicillin N (see text). **B** Production of penicillin (▲—▲) and cephalosporin (●—●) by CW-19 and the transformants TCW19.1 and TCW19.10 carrying the *pen*DE gene

**Table 3.** Transformation of *C. acremonium* CW-19 with plasmids carrying the *pcb*C or *pen*DE genes and production of benzylpenicillin by the transformants

| Vectors | Penicillin biosynthetic genes | DNA (μg) | Number of transformants obtained | Production of benzylpenicillin | Designation of penicillin-producing transformants |
|---|---|---|---|---|---|
| None | – | 0 | 0 | 0 | – |
| pULJL43 | None | 5 | 55 | 0 | – |
| pULFR10 | *pcb*C | 5 | 5 | 0 | – |
| pULFR20 | *pen*DE | 5 | 2 | 2 | TCW19.1–TCW19.2 |
| pULFR30 | IGR[a] | 5 | 9 | 0 | – |
| pULFR40 | IGR-*pen*DE | 5 | 15 | 15 | TCW19.10–TCW19.24 |

[a] IGR, intergenic region between the *pcb*C and *pen*DE genes (see Fig. 2)

*Expression of the* pen*DE gene in cephalosporin-producing strains of* C. acremonium *results in the simultaneous production of benzylpenicillin and cephalosporin*

If the *pen*DE gene of *P. chrysogenum* is expressed in cephalosporin-producing *C. acremonium*, two enzymes isopenicillin N epimerase and isopenicillin N acyltransferase would compete for the pool of isopenicillin N (Fig. 5A). *C. acremonium* CW-19, a cephalosporin-producing laboratory strain was transformed with plasmids carrying different regions of the *P. chrysogenum pcb*C-*pen*DE cluster. Transformants with each of the construc-

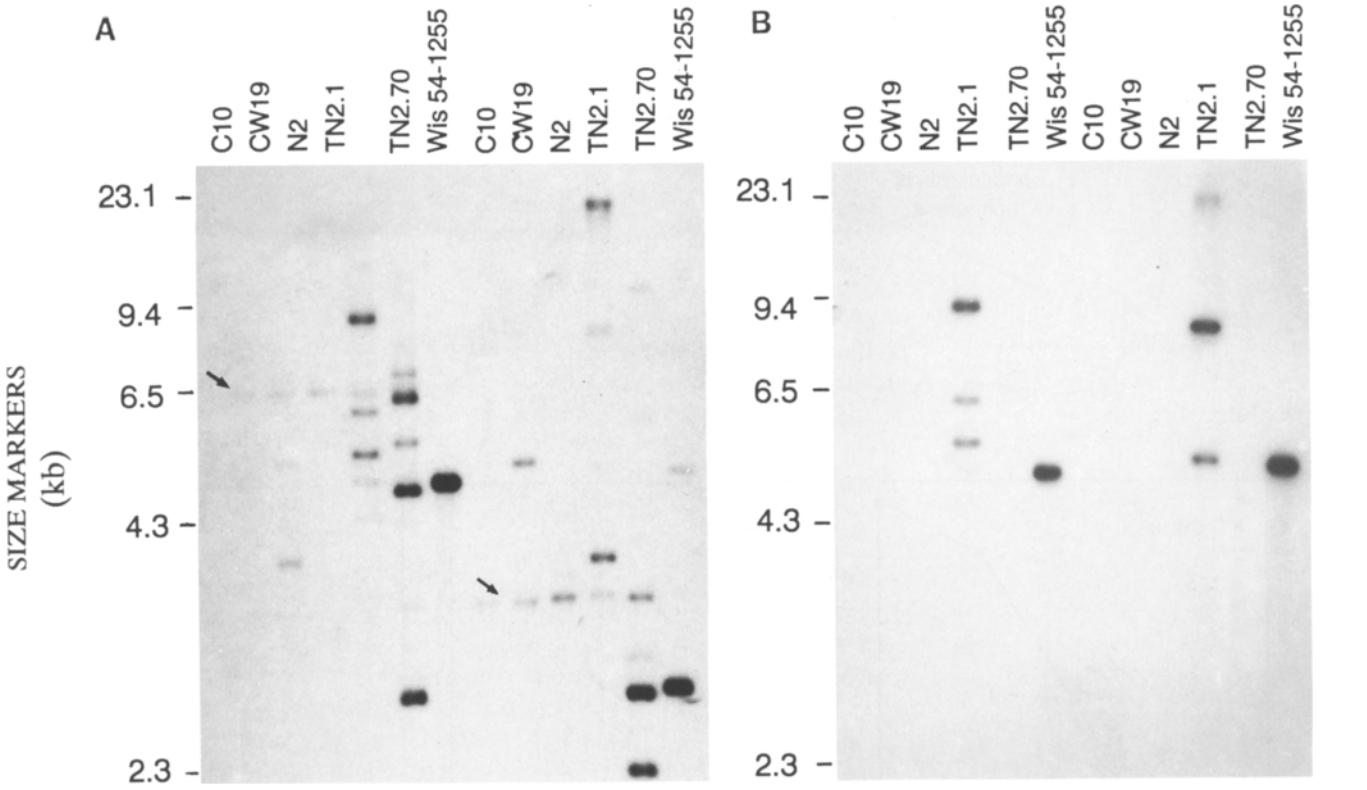


**Fig. 6 A and B.** Hybridization of *Sal*I- or *Bam*HI-digested total DNA of untransformed *C. acremonium* strains C-10, CW-19 and N2 and transformants TN2.1 and TN2.70 with probes carrying either **A** the *pcb*C gene or **B** the *pen*DE gene. DNA in lanes 1–6 was digested with *Sal*I and DNA in lanes 7–12 was digested with *Bam*HI. The *arrows* in **A** (lanes 1–3, 7–9) indicate hybridizing fragments of untransformed *C. acremonium* strains. DNA of *P. chrysogenum* Wis 54-1255 digested with either *Sal*I (lane 6) or *Bam*HI (lane 12) was used as positive (homologous DNA) control. The probe used in **A** was a 1.0 kb *Nco*I fragment that corresponds exactly to the *pcb*C gene. The probe used in **B** was the same as in Fig. 4

tions shown in Fig. 2 were obtained (Table 3) and tested for production of benzylpenicillin in solid medium using a bioassay against *M. luteus*. Two penicillin-producing transformants (one carrying the *pen*DE gene and the other with the *pen*DE gene and the upstream intergenic region) were selected and studied further in liquid cultures in comparison with the untransformed *C. acremonium* CW-19 strain. Production of cephalosporin by strain CW-19 in medium supplemented with phenylacetic acid was lower than the normal cephalosporin levels produced by this strain, apparently due to the toxic effect of phenylactic acid.

Results (Fig. 5B) indicated that whereas CW-19 produced 190 μg/ml of cephalosporin in 96 h under the conditions used, transformants TCW19.1 and TWC19.10 clearly produced lower levels of cephalosporin while at the same time they accumulated benzylpenicillin. Analysis of the levels of benzylpenicillin and cephalosporin produced by *C. acremonium* transformants TCW19.1 and TCW19.10 revealed that they produce the same total level of β-lactam antibiotic as the untransformed strain but part of the isopenicillin N intermediate is diverted toward benzylpenicillin production. The relative proportions of benzylpenicillin and cephalosporin produced by the transformants are probably determined by the substrate affinity of the two competing enzymes and vary in different transformants (see Discussion).

### *Integration of the transforming DNA in the genome of* C. acremonium

We have previously shown that transformation of *P. chrysogenum* results in the integration of the marker genes into high molecular weight DNA, which appears to be chromosomal (Cantoral et al. 1987; Díez et al. 1987). Since the DNA of *P. chrysogenum* introduced in *C. acremonium* transformants is heterologous with respect to the host DNA, it was of great interest to study the fate of the plasmid DNA after transformation. Figure 6 shows the results obtained when the total DNAs of untransformed *C. acremonium* strains C-10, CW-19 and N2 and transformants TN2.1 or TN2.70 were hybridized with probes carrying the *pcb*C gene (Fig. 6A) or the *pen*DE gene (Fig. 6B).

Results showed a faint hybridization of the *pcb*C gene (but not of the *pen*DE gene) of *P. chrysogenum* with the heterologous *pcb*C gene of untransformed *C. acremonium* strains. There was no hybridization of the corresponding lanes probed with *pen*DE even at low stringency conditions, providing evidence, for the first time, that there is no *pen*DE gene in untransformed strains of *C. acremonium*. The *Sal*I or *Bam*HI-digested DNA of transformants TN2.1 and TN2.70 gave respectively 4 and 5 bands of clear hybridization with the *pcb*C probe (other than the faint hybridizing band observed

in untransformed $N_2$). However, the sizes of the hybridizing bands were different in the DNAs of transformants obtained with plasmids pULFR50 or pULFR10. None of the bands corresponded to the expected size of the *Sal*I or *Bam*HI fragments of the transforming plasmids, which suggests that the introduced plasmid DNA has been integrated into the chromosome. The pattern of hybridization in Fig. 6A is consistent with integration at multiple sites by non-homologous recombination (see Discussion). A control hybridization with the DNA of *P. chrysogenum* Wis 54-1255 gave the expected single hybridization bands with either the *pcb*C or *pen*DE genes (Fig. 6, lanes 6 and 12).

In Fig. 6B only transformant TN2.1 (transformed with pULFR50, which carries both the *pcb*C and *pen*DE genes) gave positive hybridization. Three different positive bands were observed. Since there is no *pen*DE gene in *C. acremonium* (lanes 1–3, and 7–9) and no homology of the vector with *C. acremonium* chromosomal DNA, integration has to occur necessarily by non-homologous recombination.

## Discussion

Several genera of filamentous fungi are known to produce either cephalosporin-type β-lactams (e.g. *Cephalosporium, Paecilomyces, Scopulariopsis*) or penicillin-type antibiotics with hydrophobic side chains (e.g. *Penicillium, Aspergillus*, etc.) but none of the known β-lactam producers is able to synthesize both types of antibiotics (Elander 1983). We have searched for AAT activity in cephalosporin-producing fungi and failed to detect it (Alvarez et al. 1987). The lack of hybridization of total DNA from three different strains of *C. acremonium* with a *pen*DE probe (Fig. 6B) indicates that *C. acremonium*, and probably other cephalosporin producers (E. Montenegro, unpublished), lack the *pen*DE gene. Introduction of the *P. chrysogenum pen*DE gene into *C. acremonium* led to the synthesis of a penicillinase-sensitive, organic solvent-soluble antibiotic when the culture medium was supplemented with phenylacetic acid, the precursor of the benzylpenicillin side chain. This antibiotic was conclusively identified as benzylpenicillin by HPLC and NMR.

The *pen*DE gene of *P. chrysogenum*, which contains three introns (Barredo et al. 1989b) is correctly processed in *C. acremonium*, resulting in the formation of a single 1.15 kb transcript; this is identical in size to the transcript found in *P. chrysogenum* (Barredo et al. 1989b) and similar to that found in *A. nidulans* (E. Montenegro and J.F. Martín, unpublished). The identical size of the transcript in *P. chrysogenum* and *C. acremonium* suggests that *pen*DE is expressed in *C. acremonium* from its own promoter. *P. chrysogenum* and *C. acremonium* are unrelated taxonomically (Onions and Brady 1987) and they clearly differ in growth characteristics and cell morphology. However, it is likely that expression of *P. chrysogenum* genes from homologous promoters occurs in *C. acremonium* since the transcription signals and expression motifs appear to be conserved in filamentous fungi (Ballance 1986). Very little is known, however, about the promoters and regulatory regions of the genes involved in production of β-lactam antibiotics (Barredo et al. 1989a) and we cannot exclude a less efficient utilization of heterologous promoters.

The pre-AAT precursor is processed post-translationally in *P. chrysogenum* giving rise to two polypeptides of $M_r$ 11498 and 28461 respectively, the latter corresponding to the active mature AAT (Barredo et al. 1989b). We do not know at this time whether similar processing takes place in *C. acremonium*.

The plasmid DNA used in the transformation becomes integrated into high molecular weight DNA, since the hybridization pattern of the DNA of the transformants does not correlate with the fragments of the plasmids used in the transformation. The pattern of hybridization was different in transformants obtained with plasmids carrying distinct fragments of the *pcb*C-*pen*DE cluster. Since no sequence homologous to the *pen*DE gene of *P. chrysogenum* was found in *C. acremonium* it seems that integration occurs by non-homologous recombination as reported for other fungal genes (Yelton et al. 1984).

The production of benzylpenicillin by *C. acremonium* transformed with the *pen*DE gene has particular industrial relevance in addition to its scientific interest. As shown in this work, the availability of the genes involved in penicillin or cephalosporin biosynthesis makes it possible to convert a cephalosporin overproducer strain into a high penicillin producer. Introduction of the *pen*DE gene into a cephalosporin producer results in the splitting of the isopenicillin N pool to provide for synthesis of both penicillin and cephalosporin. The relative amounts of each antibiotic will be determined by the specific activities in the cell (which may reflect the gene copy number) and the substrate affinities of the two competing enzymes isopenicillin N epimerase and isopenicillin N acyltransferase (Fig. 5A). The $K_m$ of the *P. chrysogenum* isopenicillin N acyltransferase for isopenicillin N is 23 μM (Alvarez et al. 1987), but the $K_m$ of the epimerase of *C. acremonium* for isopenicillin N has not been determined. The isopenicillin N epimerases of *Nocardia lactamdurans* (Láiz et al. 1990) and *Streptomyces clavuligerus* (Usui and Yu 1989) have $K_m$ values for isopenicillin N of 270 μm, which suggests that both competing enzymes may not have very different affinities for isopenicillin N in *Cephalosporium*.

Strain CW-19 of *C. acremonium* is known to accumulate penicillin N (Zanca and Martin 1983; Pérez-Martínez and Peberdy 1979) apparently due to a partial deficiency in expandase activity. This may favour production of benzylpenicillin in strain CW-19 since penicillin N appears to be in equilibrium with isopenicillin N (Martín and Liras 1989) (Fig. 5A). A mutation resulting in complete inactivation of the isopenicillin N epimerase or the deacetoxycephalosporin C synthase of *C. acremonium* should result in a high production of penicillin by strains transformed with the *pen*DE gene.

*Acknowledgements.* This work was supported in part by a grant of Gist-Brocades, Delft, The Netherlands. J.L. Barredo and E.

Alvarez were granted fellowships of the Ministry of Education and Science of Spain, and B. Díez received a fellowship of the Diputación de León, Spain. We thank D. Schipper and A.M. Veenstra for carrying out the NMR studies. We acknowledge the excellent technical assistance of M.P. Puertas, M.I. Corrales and R. Barrientos.

## References

Alvarez E, Cantoral JM, Barredo JL, Díez B, Martín JF (1987) Purification to homogeneity and characterization of acylcoenzyme A:6-aminopenicillanic acid acyltransferase of *Penicillium chrysogenum*. Antimicrob Agents Chemother 31:1675–1682

Ausubel FM, Brent R, Kingston RE, Moore D, Smith JA, Seidman JG, Struhl K (1987) Current protocols in molecular biology. Wiley, New York

Ballance DJ (1986) Sequences important for gene expression in filamentous fungi. Yeast 2:229–236

Barredo JL, Cantoral JM, Alvarez E, Díez E, Martín JF (1989a) Cloning, sequence analysis and transcriptional study of the isopenicillin N synthase of *Penicillium chrysogenum* AS-P-78. Mol Gen Genet 216:91–98

Barredo JL, van Solingen P, Díez B, Alvarez E, Cantoral JM, Kattevilder A, Smaal EB, Groenen MAM, Veenstra AE, Martín JF (1989b) Cloning and characterization of the acylcoenzyme A:6-aminopenicillanic acid acyltransferase gene of *Penicillium chrysogenum*. Gene 83:291–300

Barredo JL, Díez B, Alvarez E, Martín JF (1989c) Large amplification of a 35-kb DNA fragment carrying two penicillin biosynthetic genes in high penicillin producing strains of *Penicillium chrysogenum*. Curr Genet 16:453–459

Caltrider PG, Niss HF (1966) Role of methionine in cephalosporin synthesis. Appl Microbiol 14:746–753

Cantoral JM, Díez B, Barredo JL, Alvarez E, Martín JF (1987) High frequency transformation of *Penicillium chrysogenum*. Bio/Technology 5:494–497

Carr LG, Skatrud PL, Scheetz III ME, Queener SW, Ingolia TD (1986) Cloning and expression of the isopenicillin N synthetase gene from *Penicillium chrysogenum*. Gene 48:257–266

Díez B, Alvarez E, Cantoral JM, Barredo JL, Martín JF (1987) Selection and characterization of *pyr*G mutants of *Penicillium chrysogenum* lacking orotidine-5′-phosphate decarboxylase and complementation by the *pyr*4 gene of *Neurospora crassa*. Curr Genet 12:277–282

Díez B, Barredo JL, Alvarez E, Cantoral JM, van Solingen P, Groenen MAM, Veenstra AE, Martín JF (1989) Two genes involved in penicillin biosynthesis are linked in a 5.1 kb *Sal*I fragment in the genome of *Penicillium chrysogenum*. Mol Gen Genet 218:572–576

Díez B, Gutiérrez S, Barredo JL, van Solingen P, van der Voort LHM, Martín JF (1990) The cluster of penicillin biosynthetic genes. Identification and characterization of the *pcb*AB gene encoding the α-aminoadipyl-cysteinyl-valine synthetase and linkage to the *pcb*C and *pen*DE genes. J Biol Chem 265:16358–16365

Elander RP (1983) Strain improvement and preservation of β-lactam-producing fungi. In: Demain AL, Solomon NA (eds) Antibiotics containing the beta-lactam structure. Springer-Verlag, Berlin, pp 97–146

Fincham JRS (1989) Transformation in fungi. Microbiol Rev 53:148–170

Hopwood DA, Malpartida F, Kieser HM, Ikeda H, Duncan J, Fujii I, Rudd BAM, Floss HG, Omura S (1985) Production of "hybrid" antibiotics by genetic engineering. Nature 314:642–644

Kolar M, Punt PJ, van den Hondel CAMJJ, Schwab H (1988) Transformation of *Penicillium chrysogenum* using dominant selection markers and expression of an *Escherichia coli lac*Z fusion gene. Gene 62:127–134

Láiz L, Liras P, Castro JM, Martín JF (1990) Purification and characterization of the isopenicillin N epimerase from *Nocardia lactamdurans*. J Gen Microbiol 136:663–671

MacCabe AP, Riach MBR, Unkless SE, Kinghorn JF (1990) The *Aspergillus npe*A consists of three contigous genes required for penicillin biosynthesis. EMBO J 9:279–287

Martín JF, Liras P (1989) Enzymes involved in penicillin, cephalosporin and cephamycin biosynthesis. In: Fiechter A (ed) Advances in biochemical engineering and biotechnology. Springer-Verlag, Berlin, pp 153–187

Martín JF, Ingolia T, Queener S (1990) Molecular genetics of penicillin and cephalosporin antibiotic biosynthesis. In: Leong SA, Berka R (eds) Molecular industrial mycology: Systems and applications. Marcel Dekker, New York, pp 149–195

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

Onions AHS, Brady BL (1987) Taxonomy of *Penicillium* and *Acremonium*. In: Peberdy JF (ed) *Penicillium* and *Acremonium*. Plenum, New York, pp 1–36

Pérez-Martínez G, Peberdy JF (1985) Production of cephalosporin C, and its intermediates, by raised-titre strains of *Acremonium chrysogenum*. Enzyme Microb Technol 7:389–394

Queener SW, Ingolia TD, Skatrud PL, Chapman JL, Kaster KR (1985) A system for genetic transformation of *Cephalosporium acremonium*. In: Leive L (ed) Microbiology 1985. ASM Publications, Washington, DC, pp 468–472

Ramos FR, López-Nieto MJ, Martín JF (1986) Co-ordinate increase of isopenicillin N synthetase, isopenicillin N epimerase and deacetoxycephalosporin C synthetase in a high cephalosporin-producing mutant of *Acremonium chrysogenum* and simultaneous loss of the three enzymes in a non-producing mutant. FEMS Microbiol Lett 35:123–127

Ramsden M, McQuade BA, Saunders K, Turner MK, Harford S (1989) Characterization of a loss-of-function mutation in the isopenicillin N synthetase gene of *Acremonium chrysogenum*. Gene 85:267–273

Samson SM, Belagaje R, Blankenship DT, Chapman JL, Perry D, Skatrud PL, Van Frank RM, Abraham EP, Baldwin JE, Queener SW, Ingolia TD (1985) Isolation, sequence determination and expression in *Escherichia coli* of the isopenicillin N synthetase gene from *Cephalosporium acremonium*. Nature 318:191–194

Samson SM, Dotzlaf JE, Slisz ML, Becker GW, Van Frank RM, Veal LE, Miller JR, Yeh W-K, Queener SW, Ingolia TD (1987) Cloning and expression of the fungal expandase/hydroxylase gene involved in cephalosporin biosynthesis. Bio/Technology 5:1207–1214

Shirafuji H, Fujisawa Y, Kida M, Kanzaki T, Yoneda M (1979) Accumulation of tripeptide derivatives by mutants of *Cephalosporium acremonium*. Agric Biol Chem 43:155–160

Smith DJ, Brunham MKR, Edwards J, Earl AJ, Turner G (1990) Cloning and heterologous expression of the penicillin biosynthetic gene cluster from *Penicillium chrysogenum*. Bio/Technology 8:39–41

Usui S, Yu C-A (1989) Purification and properties of isopenicillin N epimerase from *Streptomyces clavuligerus*. Biochim Biophys Acta 9:76–85

Yelton MM, Hamer JE, Timberlake WE (1984) Transformation of *Aspergillus nidulans* by using a *trp*C plasmid. Proc Natl Acad Sci USA 81:1470–1474

Zanca DM, Martín JF (1983) Carbon catabolite regulation of the conversion of penicillin N into cephalosporin C. J Antibiot 36:700–708

Communicated by C. van den Hondel