Journal of Industrial Microbiology, 9 (1992) 73–90
Elsevier

SIM 00385

Review

Clusters of genes for the biosynthesis of antibiotics: regulatory genes and overproduction of pharmaceuticals

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(Received 10 August 1991; accepted 6 October 1991)

Key words: β-Lactam antibiotics; Penicillin; Cephalosporin; Cephamycin; Biosynthetic gene clusters; Control of expression

SUMMARY

In the last decade numerous genes involved in the biosynthesis of antibiotics, pigments, herbicides and other secondary metabolites have been cloned. The genes involved in the biosynthesis of penicillin, cephalosporin and cephamycins are organized in clusters as occurs also with the biosynthetic genes of other antibiotics and secondary metabolites (see review by Martín and Liras [65]). We have cloned genes involved in the biosynthesis of β-lactam antibiotics from five different β-lactam producing organisms both eucaryotic (*Penicillium chrysogenum*, *Cephalosporium acremonium* (syn. *Acremonium chrysogenum*) *Aspergillus nidulans*) and procaryotic (*Nocardia lactamdurans*, *Streptomyces clavuligerus*). In *P. chrysogenum* and *A. nidulans* the organization of the *pcb*AB, *pcb*C and *pen*DE genes for ACV synthetase, IPN synthase and IPN acyltransferase showed a similar arrangement. In *A. chrysogenum* two different clusters of genes have been cloned. The cluster of early genes encodes ACV synthetase and IPN synthase, whereas the cluster of late genes encodes deacetoxycephalosporin C synthetase/hydroxylase and deacetylcephalosporin C acetyltransferase. In *N. lactamdurans* and *S. clavuligerus* a cluster of early cephamycin genes has been fully characterized. It includes the *lat* (for lysine-6-aminotransferase), *pcb*AB (for ACV synthase) and *pcb*C (for IPN synthase) genes. Pathway-specific regulatory genes which act in a positive (or negative) form are associated with clusters of genes involved in antibiotic biosynthesis. In addition, widely acting positive regulatory elements exert a pleiotropic control on secondary metabolism and differentiation of antibiotic producing microorganisms.

The application of recombinant DNA techniques will contribute significantly to the improvement of fermentation organisms.

BIOSYNTHESIS OF SECONDARY METABOLITES

The genetic capability of many soil microorganisms, particularly *Bacillus*, actinomycetes and filamentous fungi, and plant cells to synthesize antibiotics and other secondary metabolites is amazing [60,88]. Secondary metabolites are synthesized via multistep pathways leading from precursors (usually intermediates of primary metabolism) to the specific moieties of these metabolites [26,62]. The component moieties of secondary metabolites, activated in the form of adenylated, phosphorylated, or coenzyme A (CoA) derivatives, are finally linked together to form the final products [62]. The biosynthetic steps are carried out by enzymes that are specific for each antibiotic or secondary metabolite. However, some of the antibiotic biosynthetic enzymes (e.g. penicillin acyltransferase) have a broad substrate specificity, which gives rise to families of closely related compounds [62].

In the last few years numerous genes involved in the biosynthesis of antibiotics, pigments, herbicides, and other secondary metabolites (see reviews [38,54,61,87] including some plant-secondary metabolites have been cloned. A comprehensive picture of their organization in clusters is emerging. Understanding expression of these genes, which are not essential for growth, is of utmost importance for establishing how they have evolved as compared to operons of primary biosynthetic genes. Furthermore, knowledge of the regulatory mechanisms controlling gene expression has relevance for industrial overproduction of these metabolites [14,65].

CLUSTERING OF ANTIBIOTIC BIOSYNTHETIC AND RESISTANCE GENES

Some of the genes coding for specific enzymes involved in antibiotic biosynthesis are located in clusters on the bacterial chromosome or on plasmids [35,37,51]. These clusters of genes contain frequently one antibiotic resistance gene which avoids the suicide of the antibiotic-producing strain [65]. In eukaryotic organisms (filamentous fungi and plants) the secondary metabolites may be encoded by genes located on separate subclusters in different chromosomes.

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Genetic mapping showed that all nine classes of oxytetracycline negative (OTC) mutations map in two chromosomal clusters in *Streptomyces rimosus* [79], although more recent evidence indicates that all of them may be linked in a single cluster [9,10]. The first physical evidence of clustering of genes involved in antibiotic biosynthesis was obtained when a DNA fragment containing several genes involved in undecylprodigiosin biosynthesis was isolated [27]. A cluster of chromosomal genes involved in the four final steps of the rifamycin biosynthetic pathway and two earlier steps has also been reported [84]. The structural genes coding for two pigments (actinorhodin and undecylprodigiosin) produced by *Streptomyces coelicolor* A3(2) are located in clusters [81]. The erythromycin biosynthetic genes are also clustered [92]. A similar linkage of mutations blocking avermectin biosynthesis has been observed [47].

The genes involved in biosynthesis of the peptide antibiotics gramicidin S, tyrocidine, bialaphos, etc are also clustered. Similarly, the genes encoding biosynthesis of streptomycin appear to be in a single cluster [23–25]. Many other examples of clustering of antibiotic genes are being reported every year (see review by Martín and Liras [65]).

PENICILLIN AND CEPHALOSPORIN BIOSYNTHESIS: AN OUTLINE

The application of molecular genetics to antibiotic biosynthesis in microorganisms has increased rapidly in the last few years [36,63,64,68,76] but knowledge on the molecular genetics of industrial microorganisms is still scarce when compared with the information available on gene organization and expression in *Escherichia coli* and other 'model' microorganisms.

Penicillins, cephalosporins and cephamycins are β-lactam antibiotics formed by condensation of L-α-aminoadipic acid (an intermediate in the lysine biosynthetic pathway in fungi), L-cysteine and L-valine (see review by Martín and Liras, [64]) (Fig. 1). In cephamycin-producing actinomycetes, lysine (an amino acid belonging to the so-called aspartate family) is synthesized by the dihydrodipicolinate pathway which does not include α-aminoadipic acid as an intermediate. Therefore, this precursor is formed by catabolism of lysine by the action of lysine-6-aminotransferse [57].

The three amino acids are linked together to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) which is the first common intermediate in the biosynthetic pathways of penicillin and cephalosporin [55] (Fig. 1). In the second step, ACV is oxidatively cyclized by removal of four hydrogen atoms to form the bicyclic *penam* nucleus (a β-lactam fused to a thiazolidine ring) of isopenicillin N which is present in all penicillins. From here the pathway diverges to hydrophobic penicillins in *P. chrysogenum* and *A. nidulans* and to cephalosporins and cephamycins in various molds and actinomycetes [64,72]. Cephalosporins and cephamycins (7-α-methoxycephalosporins) contain the *cephem* bicyclic ring system (a β-lactam fused to a dihydrothiazine ring).

ACV is synthesized by a multifunctional peptide synthetase

Formation of the tripeptide ACV is carried out by the enzyme ACV synthetase. This enzyme required ATP for the reaction [5] and, therefore, should be named 'synthetase', different from the isopenicillin N synthase and deacetoxycephalosporin C synthase which do not require ATP. ACV synthesis might be the rate-limiting step in biosynthesis of penicillins and cephalosporins and is known to be regulated by glucose in *P. chrysogenum* and *N. lactamdurans* [20,78], by phosphate in *S. clavuligerus* [97] and by ammonium in *S. clavuligerus and C. acremonium* [98]. It is also strongly affected by the oxygen transfer rate of the cultures [80].

Several factors affecting the ACV synthesis in vivo have been characterized in low and high penicillin-producing cultures [56]. ACV synthesis is stimulated when protein synthesis is blocked with cycloheximide or anisomycin indicating that it is synthesized by a non-ribosomal mechanism [56]. Little information is available on enzymes involved in ACV tripeptide biosynthesis. Cell-free systems catalyzing ACV formation have been described for *C. acremonium* [5,6] and in *S. clavuligerus* [48,49]. A multifunctional peptide synthetase that catalyzes the formation of ACV has been recently purified from *A. nidulans* [90], and *C. acremonium* [3,96]. The molecular weight of the *A. nidulans* enzyme was originally reported to be 220 kDa [90], but upon further analysis appears to be larger than 400 kDa (von Döhren, personal communication).

An unusually large gene (pcbAB) encodes ACV synthetase

The gene *pcb*AB encoding the ACV synthetase of *P. chrysogenum* was cloned using two different strategies: i) complementation of mutants of *P. chrysogenum* blocked in penicillin biosynthesis (*npe*5 and *npe*10); and ii) transcriptional mapping of the regions around the previously cloned (see below) *pcb*C-*pen*DE cluster [22]. *P. chrysogenum* DNA fragments, cloned in EMBL3 or cosmid vectors, from the upstream region of the *pcb*C-*pen*DE cluster carry a gene (*pcb*AB) that complemented the deficiency of α-aminoadipyl-cysteinyl-valine synthetase of mutants *npe*5 and *npe*10, and restored penicillin production to mutant *npe*5. A protein of at least 250 kDa was observed in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gels of cell-free extracts of

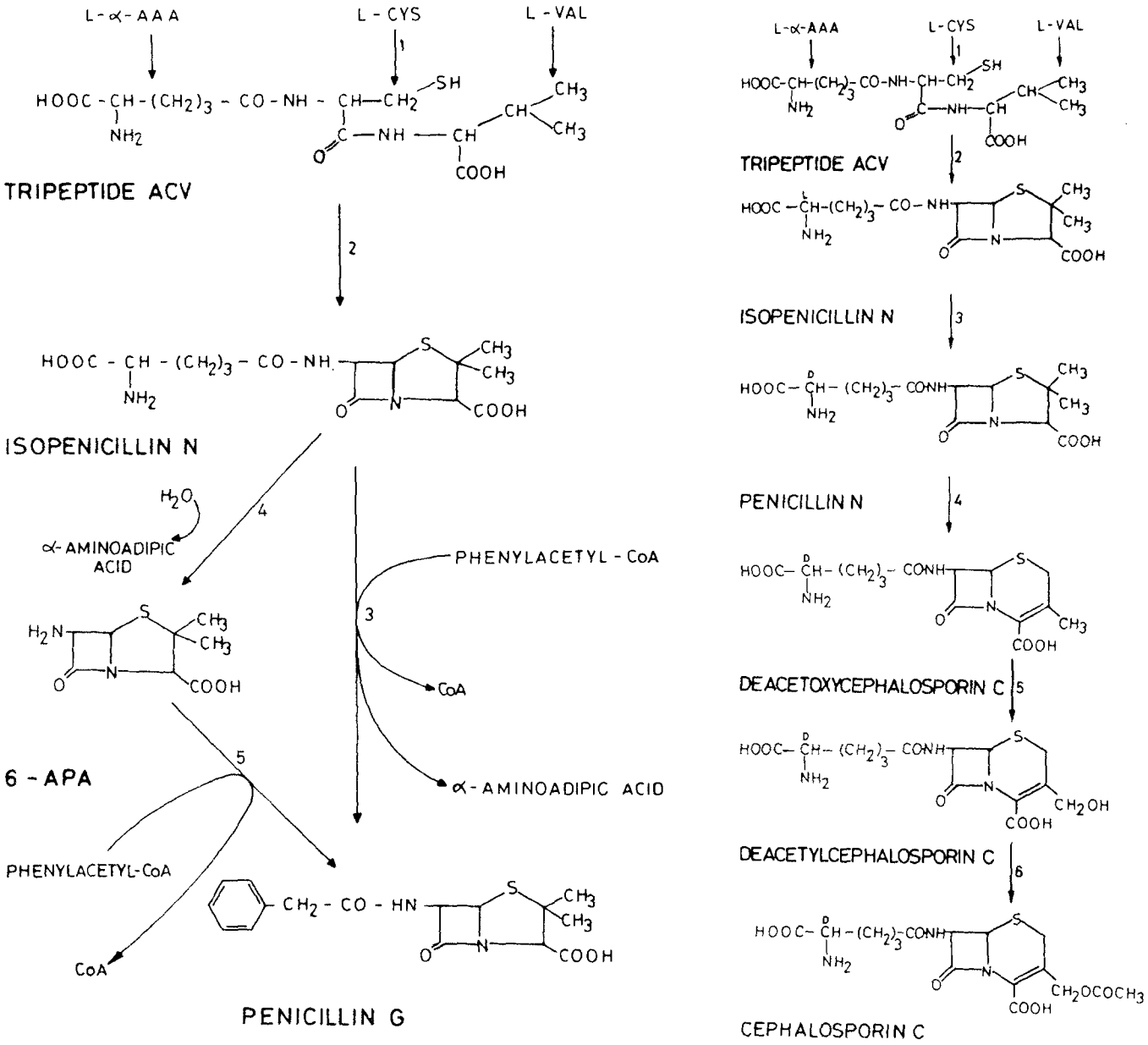


Fig. 1. **Left**: Biosynthetic pathway of penicillin G from the amino acids L-α-aminoadipic, L-cysteine and L-valine. 1, ACV synthetase; 2, isopenicillin N synthase; 3, isopenicillin N acyltransferase; 4, isopenicillin N amidase (6-APA forming); 5, 6-APA acyltransferase. **Right**: Biosynthetic pathway 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. From Martín and Liras [64].

complemented strains that were absent in the *npe*5 and *npe* 10 mutants but exists in the parental strain from which the mutants were obtained. Transcriptional mapping studies showed the presence of one long transcript of about 11.5 kb that hybridized with several probes internal to the *pcb*AB gene, and two small transcripts of 1.15 kb that hybridized with the *pcb*C or the *pen*DE gene, respectively (Fig. 2). The transcription initiation and termination regions of the *pcb*AB gene were mapped by hybridization with several small probes. The region has been completely sequenced. It includes an open reading frame of 11376 nucleotides that encodes a protein with a deduced M_r of 425971. No introns appear to occur in the *pcb*AB gene. Three repeated domains were found in the

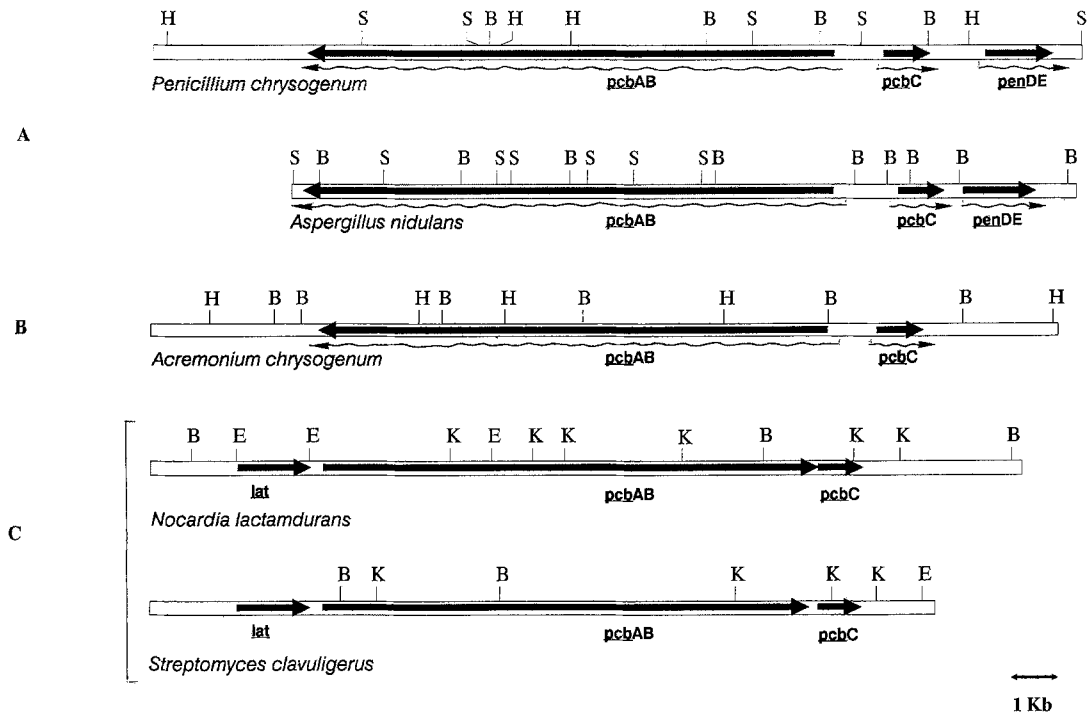


Fig. 2. Clusters of genes involved in (**A**) penicillin biosynthesis in *P. chrysogenum* and *A. nidulans*; (**B**) early steps of cephalosporin biosynthesis in *C. acremonium*; and (**C**) early steps of cephamycin biosynthesis in *N. lactamdurans* and *S. clavuligerus*. Note the different orientation of the *pcb*AB gene with respect to the *pcb*C gene in procaryotic and eucaryotic β-lactam producers. The known transcripts formed from the penicillin and cephalosporin cluster of genes are indicated by wavy lanes.

α-aminoadipyl-cysteinyl-valine synthetase that have high homology with the gramicidin S synthetase I and tyrocidine synthetase I (Fig. 3). The *pcb*AB is linked to the *pcb*C and *pen*DE genes and is transcribed in the opposite orientation to them (Fig. 2).

Initially, two *loci pcb*A and *pcb*B were allocated for the enzymatic steps that form the α-aminoadipyl-cysteine (AC) dipeptide and the ACV tripeptide [67]. However, genetic evidence [22] indicates that a single gene encodes an unusually large polypeptide which activates the three amino acids in the L form, racemizes L to D valine and carries out the polymerization steps to form the ACV tripeptide.

The pcbC gene encodes isopenicillin N synthase

The *pcb*C gene of *P. chrysogenum*, encoding the isopenicillin N synthase of *P. chrysogenum* was initially cloned from a high penicillin producing strain *P. chrysogenum* 23X-80-269-37-2 [11]. We cloned it from strain AS-P-78, in a 3.9-kb *Sal*I fragment using a probe corresponding to the amino terminal end of the enzyme. The *Sal*I fragment was trimmed down to a 1.3-kb *Nco*I–*Bgl*II fragment that contained an ORF of 996 nucleotides encoding a polypeptide of 331 amino acids with an M_r of about 38000 [7]. The predicted polypeptide encoded by the *pcb*C gene of strain AS-P-78 contains a tyrosine at position 194 whereas the gene of the high penicillin-producing strain shows an isoleucine at the same position [11]. The *pcb*C gene does not contain introns and is expressed in *E. coli* minicells using the P_L promoter of phage lambda [7].

The deduced amino acid sequence of the isopenicillin N synthase of *P. chrysogenum* is very similar to that of *Streptomyces griseus* [29], *N. lactamdurans* [19] and other actinomycetes and filamentous fungi (reviewed by Martín et al. [67]) (Fig. 4).

One of the two conserved cysteines found in all other IPNS's is absent in the *N. lactamdurans pcb*C (residue 249 of *N. lactamdurans* IPNS). This striking result was reconfirmed by sequencing both strands with *Taq* polymerase and sequenase. A GCC triplet corresponding to alanine substitutes the normal TGC triplet encoding cysteine in other *Streptomyces* and fungi.

Conservation of the amino acids in all cyclases is so high (Fig. 4) that it is difficult to conclude which are the important amino acid sequences for the mechanism of ring closure which is carried out by the cyclases. Amino acids which are important for cyclase activity have been

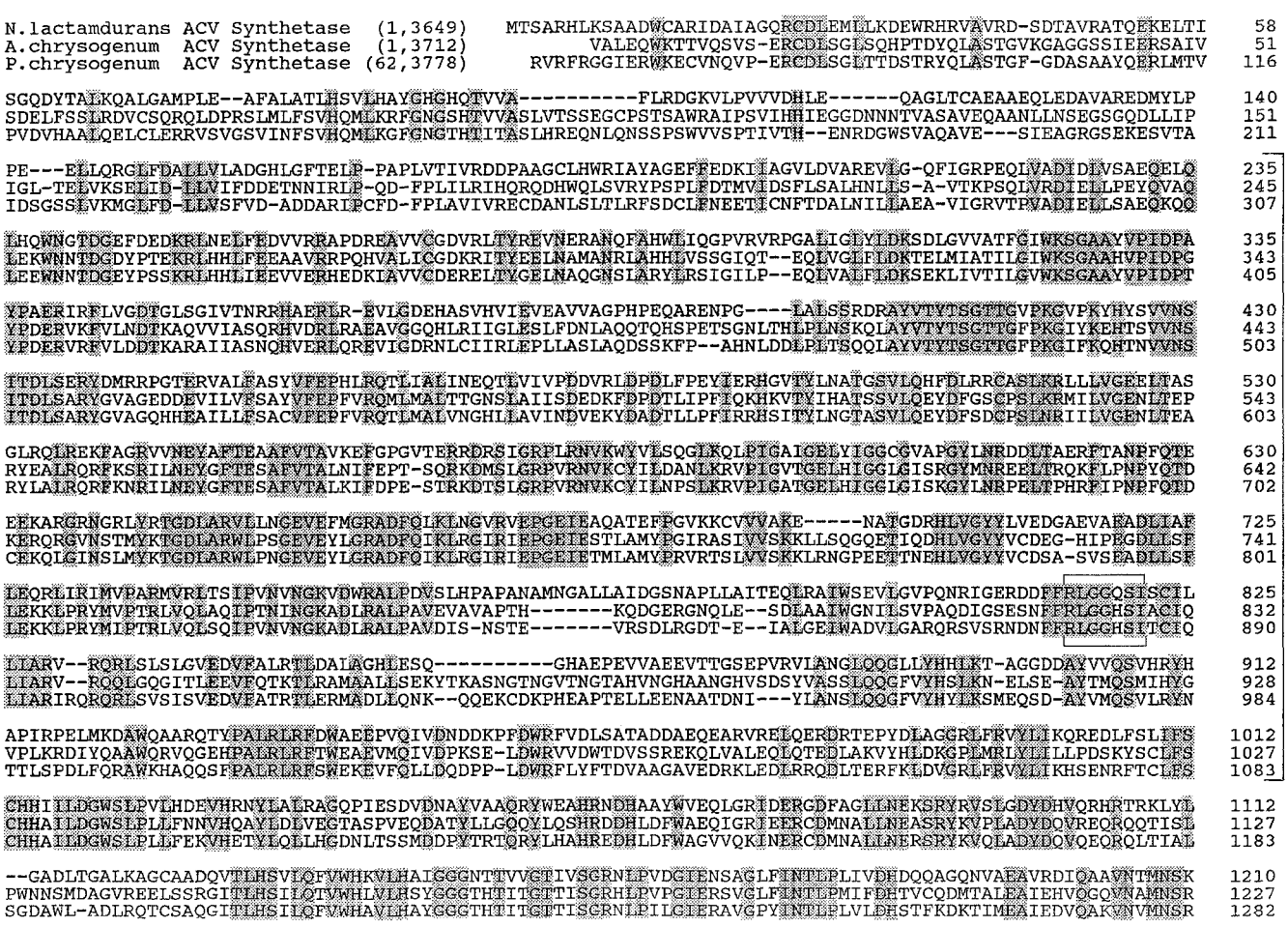


Fig. 3. Comparison of the deduced amino sequences of the ACV synthetases of *N. lactamdurans*, *C. acremonium* and *P. chrysogenum*. Some gaps have been introduced to obtain maximal alignment. The location of the three repeated domains in the three ACV synthetases is indicated by brackets on the right. Identical amino acids are shaded. The position of three putative phosphopantetheine binding sequences are boxed with thin lanes and the thioesterase active center is boxed with thick lane.

established by in vitro mutagenesis of cysteine residues to serine [82] and by characterization of a mutation in the isopenicillin N synthase gene of *C. acremonium* N_2 [76,77]. The C → T mutation at nucleotide 854 existing in strain N_2 changes amino acid 285 from proline to leucine.

Substitution of a serine for Cys-104 in the IPNS polypeptide of *C. acremonium* reduced the activity by about 95%, whereas substitution of Cys-255 has a much less dramatic effect [82], which agrees with the lack of effect of the absence of the second cysteine in the *N. lactamdurans* enzyme, that corresponds to the Cys-255 of *C. acremonium*. In this way, the cysteine at position 104 and the proline at position 285 in the *C. acremonium* cyclase (but not the cysteine at position 255) have been shown to be required for enzyme activity. Recently, a new model for the mechanism of cyclization of the ACV tripeptide at the active center of IPNS has been proposed (V.J. Chen, Abstracts of the International Symposium on '50 Years of Penicillin Utilization', Berlin, 1990) in which a histidine rather than a cysteine is used to form an initial bridge between the enzyme and the oxygen molecule via an Fe^{2+} atom (a known cofactor of the enzyme) [76]. The second cysteine, therefore, is not required for IPNS activity. In fact, the K_m of the *N. lactamdurans* IPNS is almost identical to that of the IPNS's of the other β-lactam producers [13,64].

Isopenicillin N: acyl-CoA acyltransferase is encoded by the penDE gene

In the last step of penicillin biosynthesis the α-aminoadipyl side-chain of isopenicillin N is exchanged for phenylacetic acid. This reaction, which is catalyzed by the isopenicillin N: acyl-CoA acyltransferase (IAT), occurs only in penicillin-producing strains. This enzyme

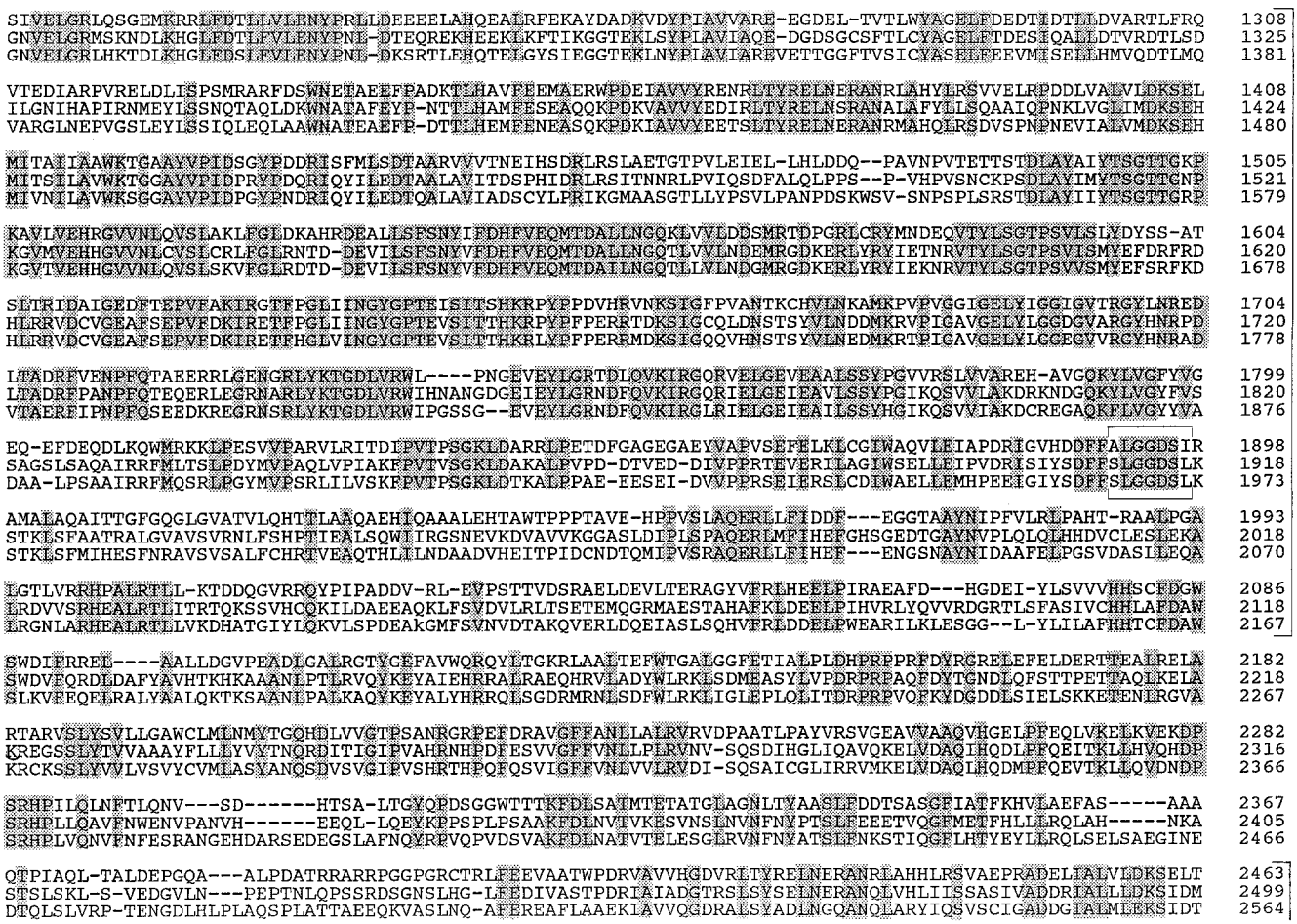


Fig. 3 (continued).

does not exist in *C. acremonium* and other cephalosporin producers [1] due to the absence of the gene [31].

The IAT (previously known as AAT) of *P. chrysogenum* has been purified to homogeneity. The purified preparation that catalyzed the formation of benzylpenicillin from phenylacetyl-CoA and 6-APA or (with a lower affinity) from IPN was shown to contain three proteins sized at ca. 40, 29 and 11 kDa. The N-terminal sequence of the 29-kDa protein was used to isolate *P. chrysogenum* DNA that contained an open reading frame with three introns [8] (Fig. 5). The deduced amino acid sequence of the open reading frame encodes a 39-kDa protein. A DNA sequence in the gene was found that corresponded to the N-terminal sequence of the 10-kDa protein, and downstream of this sequence the nucleotide sequence matched the N-terminal sequence of the 29-kDa protein [8]. The large protein (40 kDa) corresponded to a heterodimer formed from the 10- and 29-kDa subunits [94]. The 10- and 29-subunits are probably formed by proteolysis of the 39-kDa protein encoded in the cloned gene.

The gene (*pen*DE) encoding the IAT of *A. nidulans* was cloned and identified by complementation of the *npe* mutants of *P. chrysogenum* lacking IAT activity [70]. The *A. nidulans pen*DE gene is very similar to the previously cloned *pen*DE gene of *P. chrysogenum* [8] (Fig. 5). Both genes contain three introns in similar positions that were identified by comparison with the fungal consensus intron/exon splicing sequences [4] and confirmed by mRNA hybridization experiments using three oligonucleotides internal to each intron.

The presence of three introns in the *pen*DE genes suggests that they have not originated from *Streptomyces* in a recent transfer event as proposed for the other genes of the penicillin pathway. These genes appear to have evolved in *P. chrysogenum*, *A. nidulans* and other benzylpenicillin-producing fungi from ancestral fungal genes.

The cephalosporin biosynthetic pathway is encoded by two clusters of genes pcbAB-pcbC and cefEF-cefG

The availability of the *pcb*AB gene of *P. chrysogenum* for heterologous hybridization facilitated the cloning of

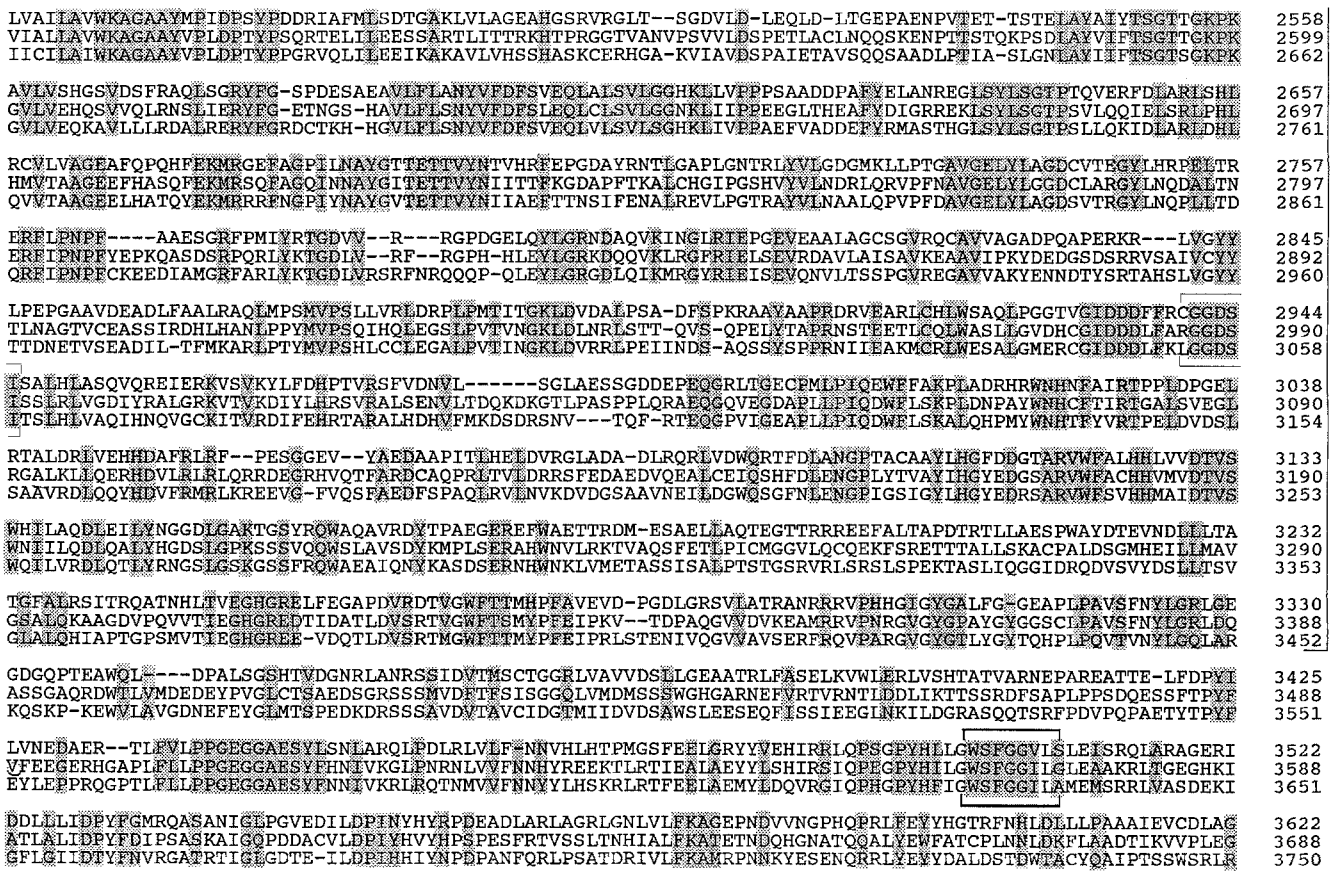


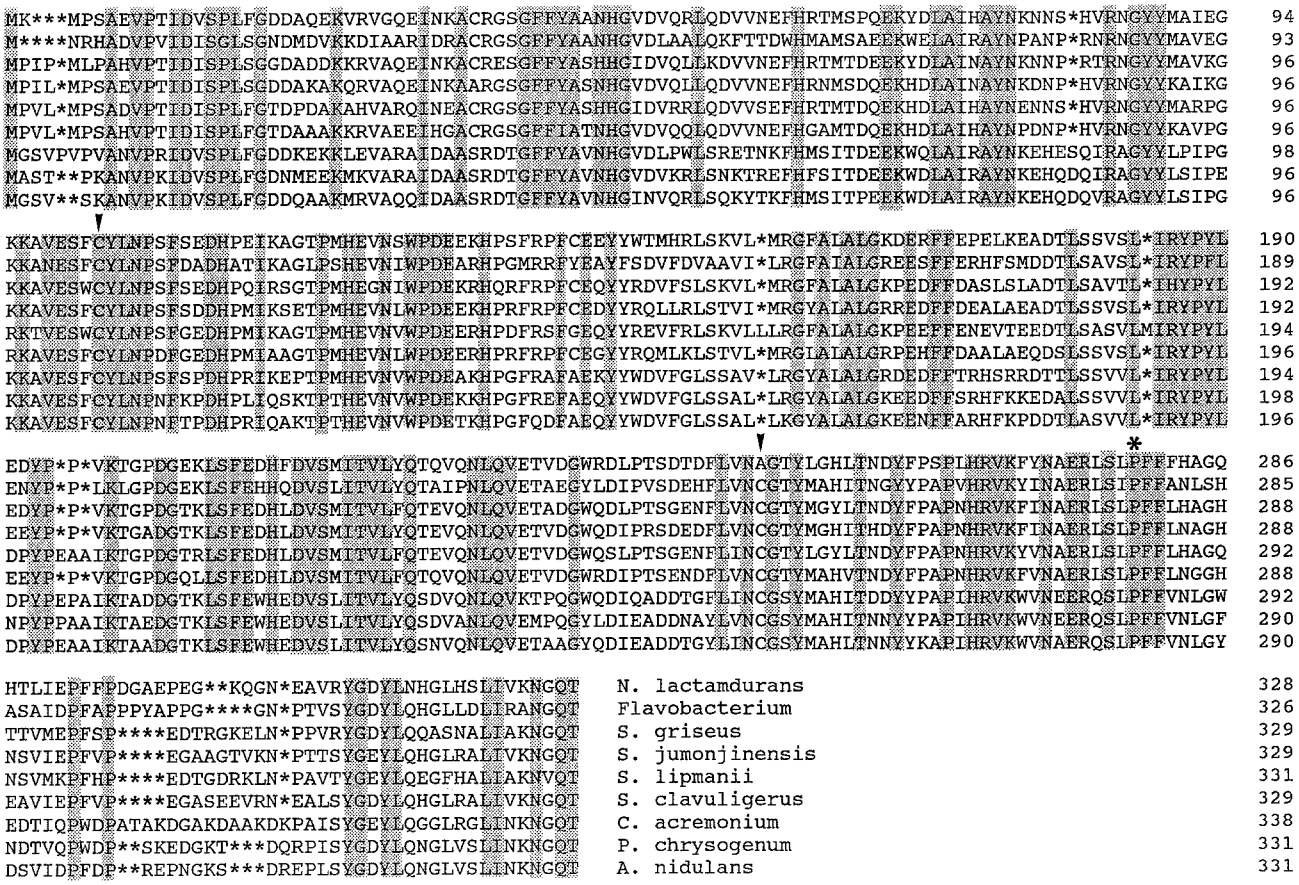
Fig. 3 (continued).

the ACV synthetases of the cephalosporin-producer *C. acremonium* and the cephamycin producer *N. lactamdurans*.

A 24-kb region of *C. acremonium* C10 DNA was cloned by hybridization with the *pcb*AB and *pcb*C genes of *P. chrysogenum* [32]. The *pcb*AB was found to be closely linked to the *pcb*C gene forming a cluster of early cephalosporin-biosynthetic genes. A 3.2-kb *Bam*HI fragment of this region complemented the mutation in the structural *pcb*C gene of the *C. acremonium* N2 mutant, resulting in cephalosporin production. A functional α-aminoadipyl-cysteinyl-valine (ACV) synthetase was encoded by a 15.6-kb *Eco*RI-*Bam*HI DNA fragment (Fig. 2), as shown by complementation of an ACV synthetase-deficient mutant of *P. chrysogenum*. Two transcripts of 1.15 and 11.4 kb were found by Northern (RNA blot) hybridization of *C. acremonium* RNA with probes internal to the *pcb*C and *pcb*AB genes, respectively. An open reading frame of 11 136 bp was located upstream of the *pcb*C gene that matched the 11.4-kb transcript initiation and termination regions. It encoded a protein of 3712 amino acids with a deduced M_r of 414 791. The nucleotide sequence of the gene showed 62.9% similarity to the *pcb*AB gene encoding the ACV synthetase of *P. chrysogenum*; 54.9% of the amino acids were identical in both ACV synthetases. Three highly repetitive regions occur in the deduced amino acid sequence of *C. acremonium* ACV synthetase. Each is similar to the three repetitive domains in the deduced sequence of *P. chrysogenum* ACV synthetase and also to the amino acid sequence of gramicidin S synthetase I and tyrocidine synthetase I of *Bacillus brevis* [52,69]. These regions probably correspond to amino acid-activating domains in the ACV synthetase protein. In addition, a thioesterase domain was present in the ACV synthetases of both fungi. The *pcb*AB gene is linked to the *pcb*C gene, forming a cluster of early cephalosporin-biosynthetic genes (Fig. 2).

A separate cluster of genes includes *cef*EF which encodes the bifunctional deacetoxycephalosporin C synthetase/hydroxylase [83] and *cef*G which encodes the acetyl-CoA : deacetylcephalosporin C acetyltransferse (S. Gutiérrez, J. Velasco and J.F. Martín, unpublished).

A



B

SIMILARITY OF THE pcbC GENE OF N. lactamdurans WITH THE HOMOLOGOUS GENES OF OTHER β-LACTAM PRODUCERS

	Nocardia lactamdurans	*Streptomyces griseus*	*Streptomyces jumonjinensis*	*Streptomyces lipmanii*	*Streptomyces clavuligerus*	*Flavobacterium* sp	*Acremonium chrysogenum*	*Penicillium chrysogenum*	*Aspergillus nidulans*
									AMINO ACIDS HOMOLOGY (%)
N. lactamdurans		72.6	77.0	71.5	75.0	59.3	57.3	57.3	59.6
S. griseus	78.8		75.7	73.9	72.0	59.8	53.6	56.0	57.2
S. jumonjinensis	80.9	82.0		69.7	81.5	60.9	60.4	58.4	59.1
S. lipmanii	78.8	82.8	82.0		70.9	55.3	55.6	54.4	57.0
S. clavuligerus	79.0	78.9	84.8	79.3		59.4	56.8	56.6	57.9
Flavobacterium sp	69.1	69.8	70.1	66.7	69.2		53.6	54.5	55.2
A. chrysogenum	66.5	69.7	70.9	69.8	67.8	67.0		76.6	74.0
P. chrysogenum	64.3	65.5	67.1	67.2	65.2	65.0	76.1		81.3
A. nidulans	63.7	63.0	64.7	65.3	63.8	69.6	71.5	76.2	

NUCLEOTIDES HOMOLOGY (%)

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      10v       20v       30v       40v       50v       60v       70v       80v       90v      100v
MLHVTCQGTPSEIGYHHGSAAKGEIAKAIDFATGLIHGKTKKTQAELEQLLRELEQVMKQRWPRYYEEICGIAKGAEREVSEIVMLNTRTEFAYGLVEARD
MLH: CQGTP EIGY:HGSAAK: IA::IDFA.:LI:GKTKKT:.EL.Q:L.:L.:V:.:RWP:YYEEI GIAKGAER:VSEIVMLNTRTEFAYGL .ARD
MLHILCQGTPFEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAKGAERDVSEIVMLNTRTEFAYGLKAARD
      10^       20^       30^       40^       50^       60^       70^       80^       90^      100^

     110v      120v      130v      140v      150v      160v      170v      180v      190v      200v
GCTTVYCKTPNGALQGQNWDFFTATKENLIQLTICQPGLPTIKMITEAGIIGKVGFNSAGVAVNYNALHLHGLRPTGLPSHLALRMALESTSPSEAYEKIV
GCTT.YC: PNGALQGQNWDFF:ATKENLI:LTI Q:GLPTIK.ITEAGIIGKVGFNSAGVAVNYNALHL:GLRPTG:PSH:ALR:ALESTSPS:AY::IV
GCTTAYCQLPNGALQGQNWDFFSATKENLIRLTIRQAGLPTIKFITEAGIIGKVGFNSAGVAVNYNALHLQGLRPTGVPSHIALRIALESTSPSQAYDRIV
     110^      120^      130^      140^      150^      160^      170^      180^      190^      200^

     210v      220v      230v      240v      250v      260v      270v      280v      290v      300v
SQGGMAASAFIMVGNAHEAYGLEFSPISLCKQVADTNGRIVHTNHCLLNHGPSAQELNPLPDSWSRHGRMEHLLSGFDGTKEAFAKLWEDEDNYPLSICRA
.QGGMAASAFIMVGN:HEA:GLEFSP.S: KQV D:NGR:VHTNHCLL:HG :.:EL:PLPDSW:RH RME LL.GFDGTK:AFA:LW.DEDNYP:SICRA
EQGGMAASAFIMVGNGHEAFGLEFSPTSIRKQVLDANGRMVHTNHCLLQHGKNEKELDPLPDSWNRHQRMEFLLDGFDGTKQAFAQLWADEDNYPFSICRA
     210^      220^      230^      240^      250^      260^      270^      280^      290^      300^

   310v      320v      330v      340v      350v
YKEGKSRGSTLFNIVFDHVGRKATVRLGRPNNPDETFVMTFSNLDTKSAIQANIX   Aspergillus nidulans ATCC 28901
Y.EGKSRG:TLFNI::DH. R.ATVRLGRP.NPDE FVM F.: D.:SA::A.:X
YEEGKSRGATLFNIIYDHARREATVRLGRPTNPDEMFVMRFDEEDERSALNARLX   Penicillium chrysogenum AS-P-78
   310^      320^      330^      340^      350^
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Fig. 5. Comparison of the amino acid sequences of the acyl-CoA : 6-amino penicillanic acid transferases of *A. nidulans* (upper line) and *P. chrysogenum* (lower) showing the identical or functionally conserved (:,.) amino acids (center line). The conserved 11 amino acid sequence surrounding the processing (vertical arrow) of the *P. chrysogenum* enzyme is underlined.

*cef*EF (and probably other late genes of the pathway) was located in chromosome II, whereas the *pcb*C gene was found in chromosome VI [85]. It seems that most genes of the cephalosporin-biosynthetic pathway are clustered into two groups. *pcb*AB and *pcb*C belong to the cluster of early biosynthetic genes, and *cef*EF and *cef*G are located in the second (or late) cluster of genes.

Introduction of the penDE of P. chrysogenum into C. acremonium leads to production of benzylpenicillin

C. acremonium lacks isopenicillin N acyltransferse [1]. No DNA sequence homologous to the *pen*DE gene of *P. chrysogenum* was found in the genome of three different strains of *C. acremonium*. The *pcb*C-*pen*DE gene cluster of *P. chrysogenum* complemented the isopenicillin N synthase deficiency of *C. acremonium* mutant N_2 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 [31]. When the *pen*DE gene was introduced in a cephalosporin producing strain, the total titre of β-lactam antibiotics was divided, thus forming distinct proportions of penicillin and cephalosporin in different transformants.

Fig. 4. (**A**) Comparison of the deduced amino acid sequences of the isopenicillin N synthetase of *N. lactamdurans*, *Flavobacterium sp.*, *S. griseus*, *S. jumonjinensi*, *S. lipmanii*, *S. clavuligerus*, *C. acremonium*, *P. chrysogenum* and *A. nidulans*. Identical amino acids are shaded. The position of the two conserved cysteines are indicated by arrowheads. Note that the second cysteine (position 255) is missing in the *N. lactamdurans* gene. The proline at position 285 (in the *C. acremonium* IPNS) indicated by an asterisk is essential for enzyme activity. (**B**) Percentage of similarity of the *pcb*C genes from different procaryotic and eucaryotic organism (from Coque et al. [19]).

The *pen*DE gene of *P. chrysogenum*, which contains three introns [8] is correctly processed in *C. acremonium* resulting in the formation of a single 1.15-kb transcript that is identical in size to the transcript found in *P. chrysogenum* [8] 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 the *pen*DE is expressed in *C. acremonium* from its own promoter. Although *P. chrysogenum* and *C. acremonium* are unrelated taxonomically [74] and clearly differ in growth characteristics and cell morphology, it is likely that expression of *P. chrysogenum* genes occurs in *C. acremonium* since the transcription signals and expression motifs appear to be conserved in filamentous fungi [4]. Very little is known, however, about the promoters and regulatory regions of the genes involved in β-lactam antibiotics [7] and we can not exclude a less efficient utilization of heterologous promoters.

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 homologous sequence 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 [95].

The production of benzylpenicillin by *C. acremonium* transformed with the *pen*DE gene has a great industrial relevance in addition to the scientific interest. 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 in a cephalosporin-producer results in the splitting of the isopenicillin N pool to form 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. The K_m of the *P. chrysogenum* isopenicillin N acyltransferase for isopenicillin N is 23 μM [1], but the K_m for isopenicillin N of the epimerase of *C. acremonium* has not been determined. The isopenicillin N epimerases of *N. lactamdurans* [53] and *S. clavuligerus* [89] have K_m values for isopenicillin N of 2.4 μM, which suggests that both competing enzymes may not have very different affinities for isopenicillin N in *Cephalosporium*.

Procaryotic β-lactam producers contain a lat-pcbAB-pcbC cluster with different organization than in filamentous fungi

β-Lactam antibiotics are produced by a variety of procaryotic microorganisms (actinomycetes and unicelular Gram-positive and Gram-negative bacteria including species of the genera *Streptomyces*, *Nocardia*, *Pseudomonas*, *Agrobacterium*, *Serratia*, *Gluconobacter*, *Flavobacterium* and *Xanthomonas*) [17,99]. Indeed, a 34-kb fragment of the *N. lactamdurans* DNA carrying the cluster of early cephamycin biosynthetic genes was cloned in λEMBL3 [18] by hybridization with probes internal to the *pcb*AB and *pcb*C genes of *P. chrysogenum* and *S. griseus* [29]. The *pcb*AB and *pcb*C genes were found to be closely linked together in the genome of *N. lactamdurans* (Fig. 2). The *pcb*AB gene of *N. lactamdurans* showed the same orientation as the *pcb*C gene in contrast to the divergent expression of the genes in the *pcb*AB-*pcb*C cluster of *P. chrysogenum* and *C. acremonium* (Fig. 2). The *pcb*AB gene encodes a large (3649 amino acids) multidomain δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase with a deduced M_r of 404134. This enzyme contains three repeated domains and a consensus thioesterase active center sequence as occurs also in the fungal ACV synthetases. The *pcb*C gene encodes a protein of 328 amino acids with a deduced M_r of 37469, similar to other isopenicillin N synthetases except that it lacks one of two cysteine residues conserved in all other isopenicillin N synthetases (Fig. 4).

A gene (lat) encoding lysine-6-aminotransferase which forms α-aminoadipic acid, is located in the cluster of cephamycin biosynthetic genes

Little information is available on the location of genes involved in the conversion of intermediates of primary metabolism into specific precursors of antibiotics. Only a few of those enzymes, i.e. PABA synthase [30], valine dehydrogenase [71], threonine dehydratase [91], which are involved in the formation of precursors of macrolide antibiotics, have been characterized. They were believed to be associated with primary metabolism and, therefore, the location in the chromosome of the genes encoding these enzymes with respect to the clusters of genes involved in antibiotic biosynthesis, was unclear. α-Aminoadipic acid is a specific precursor of β-lactam antibiotics, which is formed in actinomycetes by deamination of lysine by the enzyme lysine 6-aminotransferase (LAT) [50,57]. α-Aminoadipic acid is condensed with L-valine and L-cysteine to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the action of the enzyme ACV synthetase.

A gene (*lat*) encoding a lysine 6-aminotransferase has been found upstream of the *pcb*AB (encoding α-aminoadipyl-cysteinyl-valine synthetase) and *pcb*C (encoding

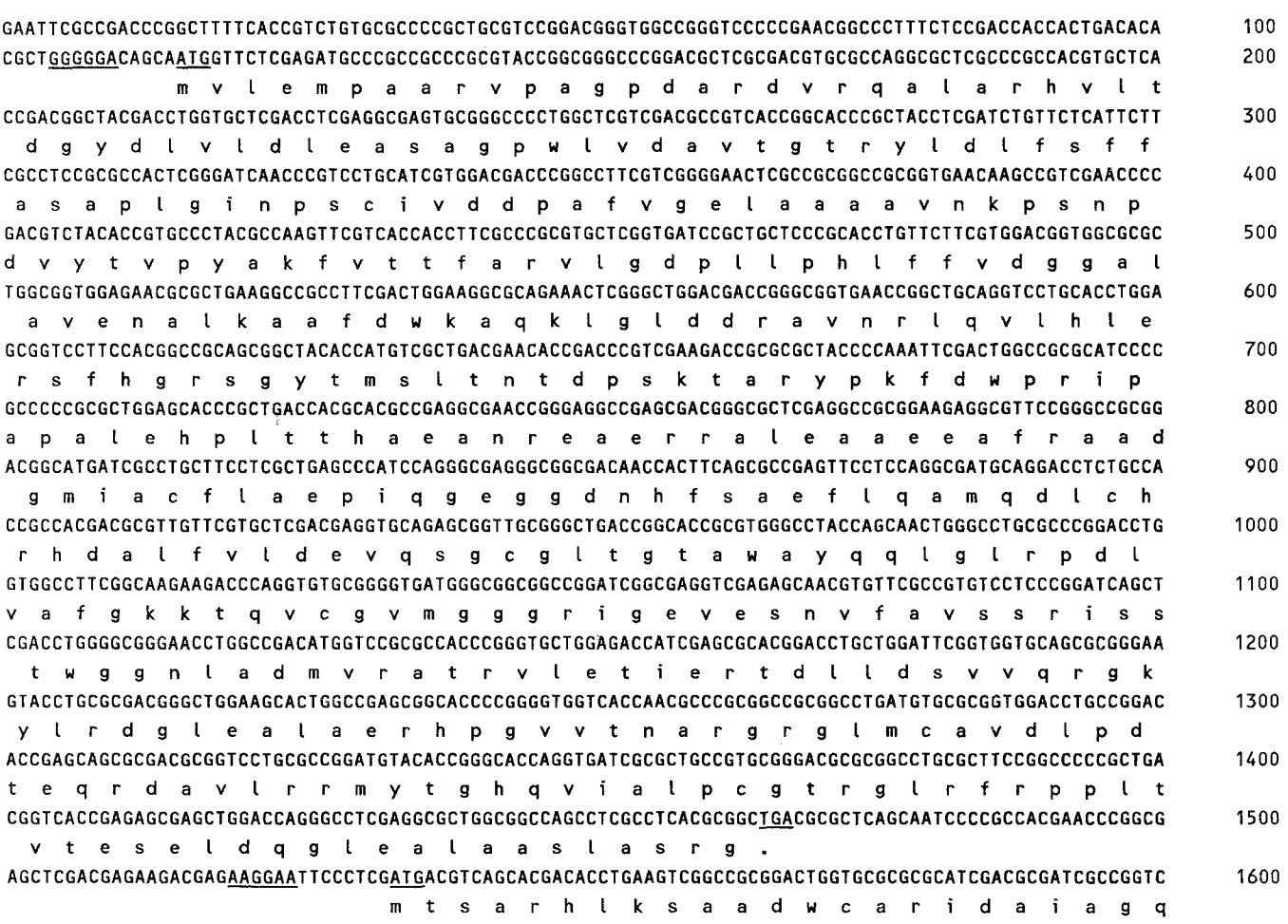

Fig. 6. Nucleotide and deduced amino acid sequence of a 1600-bp DNA fragment containing the *lat* gene and the upstream and downstream regions. The ATG initiation triplets of the *lat* and *pcb*AB genes and the TGA termination codon of the *lat* gene are boxed. Putative ribosome binding sites preceeding the ATG initiation triplets of both genes are underlined. Note the small intergenic region between the *lat* and *pcb*AB genes.

isopenicillin N synthase) genes in the cluster of early cephamycin biosynthetic genes in *N. lactamdurans* [18]. The *lat* gene was separated by a small intergenic region of 64 bp from the 5′ end of the *pcb*AB gene (Fig. 6). The *lat* gene contained an open reading frame of 1353 nucleotides (71.4% G + C) encoding a protein of 450 amino acids with a deduced molecular weight of 48 811 Da. Expression of DNA fragments carrying the *lat* gene in *Streptomyces lividans* led to a high lysine 6-aminotransferase activity which was absent from untransformed *S. lividans*. The enzyme was partially purified from *S. lividans* (pULBS8) and showed a molecular weight calculated by Sephadex gel filtration and polyacrylamide gel electrophoresis of 52 800 Da [18]. DNA sequences which hybridize strongly with the *lat* gene of *N. lactamdurans* occurred in four cephamycin-producing actinomycetes (*S. clavuligerus* NRRL3585, *S. griseus* NRRL3851, *S. lipmanii* NRRL3584 and *S. cattelya* NRRL8037), but not in four other actinomycetes which are not known to produce β-lactams (*S. albus*, *S. lividans* JI1326, *S. coelicolor* A3(2) and *S. griseus* IMRU3570), suggesting that the gene is specific for β-lactam biosynthesis and is not involved in general lysine catabolism. The protein encoded by the *lat* gene showed similarity to ornithine-5-aminotransferases and N-acetylornithine-5-aminotransferases, and contains a pyridoxal phosphate-binding consensus amino acid sequence around the Lys^{300} of the protein [18] (Fig. 7).

A similar lysine-6-aminotransferase gene has been cloned and found to be associated with the cluster of cephamycin biosynthetic genes in *S. clavuligerus*, another cephamycin-producing actinomycete ([58]; Hong, Y., Coque, J.J.R., Liras, P., Piret, J. and Martín, J.F., unpublished data) indicating that this gene is conserved in cephamycin biosynthetic clusters.

```
LAT N. lactamdurans (1-450)    MVLEMPAARVPAGPDARDVRQALARHVLTDGYD-LVLDLEASAGP--WLVDAVTGTRYLDLFSFFASAP    66
OAT Yeast           (1-422)                MSEATLSKQTIEWENKYSAHNYHPLPVVFHKAKGAHLW--DP-EGKLYLDFLSAYSAVN    56
OAT man            (34-441)                VQGPPTSDDIFEREYKYGAHNYHPLPVALERGKGIYLW--DV-EGRKYFDFLSSYSAVN    89
OAT rat            (34-439)                EQGPPSSEYIFERESKYGAHNYHPLPVALERGKGIYMW--DV-EGRQYFDFLSAYGAVS    89
ACOAT yeast         (9-428)                TSSRRFTSILEEKAFQVTTYSRPEDLCITRGKNAKLYD--DV-NGKEYIDFTAGI-AVT    63
ACOAT E. coli       (1-406)                 MAIEQTAITRATFDEVILPIYAPAEFIPVKGQGSRIW--DQ-QGKEVVDFAGGI-AVT    54

LG-INPSCIVDDPAFVGELAAAAV-NKPSNPDVYTVPYAKFVTTFARV-------LGDPLLPHLFFVDAGALAVENALKAAFDWKAQKLGL-DDRAVNRL   156
QGHCHPH-II--KALTEQAQTLTLSSRHFANDVYA-QFAKFVTEFSG--------F-ETVLP----MNTGAEAVETALKLARRWGYMKKNIPQDKAI---   136
QGHCHPK-IV--NALKSQVDKLTLTSRAFYNNVLG-EYEEYITKLF-N-------Y-HKVLP----MNTGVEAGETACKLARKWGYTVKGIQKYKAK---   169
QGHCHPK-II--EAMKSQVDKLTLTSRAFYNNVLG-EYEEYITKLF-N-------Y-NKVLP----MNTGVEAGETACKLARRWGYTVKGIQKYKAK---   169
ALGHANP-KV-AEILHHQANKLVHSSNLYFTKEC--LDLSEKIVEKTKQFGGQHDA-SRVFL----CNSGTEANEAALKFAKKHGIMKNPS---KQG---   140
ALGHCHP-AL-VNALKTQGETLWHISNV-FTNEPA-LRLGRKLIEATF-------AERVVFM-----NSGTEANETAFKLAR-HYACVRHSPF-KTK---   133

QVLHLERSFHGRSGY-TMSL-TNTDPSKTARYPKFDWPRIPAPALEHPLTTHAEANREAERRALEAAEEAFRAADGMIACFLAEPIQGEGGDNHFSAEFL   254
-ILGAEGNFHGRT-FGAISLSTDYEDSKL-HFGPF----VPNVASGHSVHKIRYGHAEDFVPILESPE-G-KNVAAII---L-EPIQREAGIVVPPADYF   223
-IVFAAGNFWGRT-LSAISSSTDPT-SYD-GFGPF----MP----GFDII--PYNDLPALERALQDP----NVAAFMV-----EPIQGEAGVVVPDPGYL   246
-IVFAVGNFWGRT-LSAVSSSTDPT-SYD-GFGPF----MPGFE---TI---PYNDLPALERALQDP----NVAAFMV-----EPIQGEAGVIVPDPGYL   246
-IVAFENSFHGRT-MGALSV-TWNS-KYRTPFGDL----VP------HVSFLNLNDEMTKLQSYIETK-K-DEIAGLI----VEPIQGEGGVFPVEVEKL   220
-IIAFHNAFHGRS-LFTVSV-GGQP-KYSDGFGPK----PA------DIIHVPFND----LHAVKAVM-D-DATCCAVV---VEPIQGEGGVTAATPEFL   209

QAMQDLCHRHDALFVLDEVQSGCGLTGTAWAYQQL--GLRPDIVAFGKKTQVCGVMGGG-RIGEVES-NVFAVSSRISSTWGGN-LADMVRATRVLETIE   349
PKVSALCRKHNVLLIVDEIQTGIGRTGELLCYDHYKAEAKPDIVLLGKALSG-GVLPVSCVLSSHDIMSCFTPGSH-GSTFGGNPLASRV-AIAALEVI-   319
MGVRELCTRHQVLFIADEIQTGLARTGRW--LAVDYENVRPDIVLLGKALSG-GLYPVSAVLCDDDIMLTIKPGEH-GSTYGGNPLGCRV-AIAALEVLE   341
TGVRELCTRHQVLFIADEIQTGLARTGRW--LAVDHENVRPDIVLLGKALSG-GLYPVSAVLCDDDIMLTIKPGEH-GSTYGGNPLGCRI-AIAALEVLE   341
TGLKKICQDNDVIVIHDEIQCGLGRSGKLWAHAYLPSEAHPDIFTSAKALGN-G-FPIAATIVNEKVNNALRVGDH-GTTYGGNPLACSV-SNYVLDTIA   326
QGLRELCDQHQALLVFDEVQCGMGRTGDL--FAYMHYALAPDILTSAKALGG-G-FPISAMLTTAEIASAFHPGSH-GSTYGGNPLACAV-AGAAFDIIN   303

RTDLL-DSVVQRG-KYLRDGLEALAERHPGVVTNARGRGLMCAVDL-P-DTEQRDA-VLRRMYTGHQVIALPCGTRGLRFRPPLTVTESEIDQGLEALAA   444
RDEKLCQRAAQLGSSFIAQ-LKALQAKSNGIISEVRGMGLLTAIVIDPSKANGKTAWDLCLLMKDQGLLAKPTHDHIIRLAPPLVISEEDLQTGVETIAK   418
EENLAENADKLGIILRNEL-MKLPSDVVTA----VRGKGLLNAIVIKETKDWD--AWKVCLRLRDNGLLAKPTHGDIIRFAPPLVIKEDEIRESIEIINK   434
EEHLAENADKMGAILRKEL-MKLPSDVVTA----VRGKGLLNAIVIRETKDCD--AWKVCLRLRDNGLLAKPTHGDIIRLAPPLVIKEDEIRESVEIINK   434
DEAFLKQVSKKSDILQKRL-REIQAKYPNQIKT-IRGKGLMLGAE----FVEP--PTEVIKKARELGLLIITAGKSTVRFVPALTIEDELIEEGMDAFEK   421
TPEVLEGIQAKRQRFVDHL-QKIDQQY-DVFSD-IRGMGLLIGAELKPQYKGR--ARDFLYAGAEAGVMVLNAGPDVMRFAPSLVVEDADIDEGMQRFAH   398

SIASRG      450
CIDLL       422
TILSF       441
TILSF       439
AIEAVYA      428
AVAKVVGA     406
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Fig. 7. Comparison of lysine 6-aminotransferase with ornithine aminotransferases and N-acetyl aminotransferase (see text for details). Some gaps have been introduced to obtain maximal alignment of the amino acids. Identities and conservative replacements have been shaded. Conservative replacements are R–K, D–E, S–T, G–A, F–Y and I–L–V–M. Numbers at the end of the lines refer to the position in the original sequence. The Lys^{300} is indicated by an asterisk (from [18]).

The *lat* gene is closely linked to the *pcb*AB and *pcb*C genes (Figs. 2 and 6) which encode the first two steps of the cephamycin biosynthetic pathway [18]. Expression of these three genes and other late genes of the pathway is probably coordinated [12]. We are studying at present whether this coordinated regulation proceeds through formation of a single polycistronic mRNA. The lack of recognizable transcription termination signals between *lat*, *pcb*AB and *pcb*C genes supports this hypothesis.

Synthesis of α-aminoadipic acid in a coordinated form with its conversion to α-aminoadipyl-cysteinyl-valine by the ACV synthetase is clearly advantageous for the cephamycin-producing strains. The evolutionary mechanisms by which these genes have become linked is intriguing. Once they were linked together by DNA reorganization events they probably remained clustered because of the ecological advantage that antibiotic production confers to the producing strain [64].

HAVE β-LACTAM GENES BEEN TRANSFERED FROM PROCARYOTIC TO EUCARYOTIC β-LACTAM PRODUCERS?

It has been proposed that there has been a horizontal transfer of genes encoding cephalosporin production from a cephamycin-producing bacteria to cephalosporin- and penicillin-producing fungi [17,75,93].

If the transfer of β-lactam biosynthetic genes has occurred horizontally as a cluster by a single transfer event, a similar organization should be conserved in the bacteria where the genes were supposed to originate and in filamentous fungi. However, the organization of *pcb*C and *pcb*AB genes in filamentous fungi is different from that in *N. lactamdurans* and *S. clavuligerus* [19]. An intriguing question is whether the *lat* gene has been also transferred (without introns) to *Penicillium*, *Cephalosporium* and other β-lactam producing fungi, as occurs

with the *pcb*AB and *pcb*C genes. Initial evidence (C. Esmahan and J.F. Martín, unpublished) indicates that a lysine : 6-aminotransferase activity is also present in *P. chrysogenum* and *C. acremonium* but whether the gene involved is similar to the *lat* gene of *Streptomyces* and any possible role of this enzyme activity in biosynthesis of β-lactam antibiotics by fungi remains to be elucidated.

However, the possibility of DNA rearrangements following horizontal gene transfer from bacteria to fungi cannot be excluded [19].

PATHWAY-SPECIFIC REGULATORY GENES ARE ASSOCIATED WITH CLUSTERS OF GENES INVOLVED IN ANTIBIOTIC BIOSYNTHESIS

In addition to the genes encoding biosynthetic enzymes, the biosynthetic clusters contain regulatory genes (Table 1).

In most cases the regulatory gene products appear to act in a positive form inducing antibiotic biosynthesis. In *S. coelicolor*, the regulatory gene defined by class II mutants, located in the middle of the *act* cluster, is believed to code for a positive regulator of actinorhodin biosynthesis since it causes overproduction of actinorhodin when introduced into an Act$^+$ strain [39]. A 30–40-fold increase in titre was observed with an extra copy of the *act*II gene [40]. Similarly, the product of the *str*R gene of *S. griseus* (a protein of 37 kDa) is required as a positive effector for full expression of the *str*A (*aph*) encoding an aminoglycoside phosphotransferase and *str*B [23–25].

The products of the *brp*A of *Streptomyces hygroscopicus* and the product of the *dnr*K of *Streptomyces peuceticus* appear to be also positive effectors that activate transcription of the *bar* (bialaphos resistance) gene and six other genes of the bialaphos biosynthetic pathway [2], and the production of daunorubicin, respectively [46].

In one case, that of methylenomycin in *S. coelicolor*, regulation seems to be negative. Deletion of DNA or disruption of the cluster at one end gave rise to marked increase of methylenomycin [15,28]. Introduction of the whole *mmy* cluster minus the regulatory region, on a low copy number plasmid, results in an increase of production of methylenomycin (L. Woodburn cited by Chater [14]).

Other reported examples of positive effectors (e.g. the

TABLE 1

Positive or negative pathway specific regulatory genes in clusters of secondary metabolite genes

Gene	Effector	*Strain*	Secondary metabolite	Function
*brp*A	Positive	*Streptomyces hygroscopicus*	Bialaphos	Activates transcription of the *bar* (bialaphos resistance) gene and six other *bap* (biosynthetic genes)
Unnamed	Positive	*Streptomyces fradiae*	Tylosin	Activates expression of *tyl*F (encoding MOMT) and other *tyl* biosynthetic genes
*act*II	Positive	*Streptomyces coelicolor*	Actinorhodin	Increases actinorhodin production 30–40 fold in *act* strains
mmy	Negative	*Streptomyces coelicolor*	Methylenomycin	Insertional inactivation of the gene causes overproduction of methylenomycin; deletion of the region also results in over-production
*str*R	Positive	*Streptomyces griseus*		Required as a positive effector for full expression of *str*A (*aph*) and *str*B. Encodes a protein of 37 kDa
*red*D		*Streptomyces coelicolor*		Increases undecylprodigiosin biosynthesis
*dnr*R		*Streptomyces peuceticus*		Increases 10× the production of daunorubicin
*cef*R		– *Streptomyces cattleya* – *Streptomyces clavuligerus* – *Nocardia lactamdurans*		Control cephamycin biosynthesis

stimulation of cephamycin biosynthesis in *Streptomyces lactamgens* by a large fragment of *Streptomyces cattleya* DNA might be due to amplification of an hypothetical *cef*R regulatory gene, or simply to a gene-dosage effect [16].

There is a great potential industrial benefit from the understanding and utilization of regulatory DNA sequences, specially if some of these genes act on heterologous sets of genes encoding other pathways for related antibiotics. Interestingly, some of the already cloned genes may act on heterologous clusters. DNA from clusters for production of granaticin or milbemycin stimulated actinorhodin production by an *act*II mutant of *S. coelicolor* [14]. Complementation of a mutant blocked in a regulatory gene may be a useful instrument for cloning other regulatory genes.

WIDELY ACTING POSITIVE REGULATORY ELEMENTS: PLEIOTROPIC EFFECTORS CONTROL SECONDARY METABOLISM AND DIFFERENTIATION

Secondary metabolism and morphological differentiation are closely linked in *Streptomyces* and other soil microorganisms. In fact, secondary metabolism may be understood as a form of biochemical differentiation. A factor (2-isocapryloyl-3-hydroxymethyl-τ-butyrolactone) is a pleiotropic regulatory substance that controls streptomycin biosynthesis and resistance and sporulation in *S. griseus* and *Streptomyces bikiniensis* at concentrations as low as 10^{-9} M [33]. In *S. griseus*, streptomycin production requires not only an active pathway-specific positive regulatory gene *str*R (see above) [73] but also synthesis of A-factor.

A gene encoding A-factor production (*afs*A) was cloned from the total DNA of *S. bikiniensis* [42,45]. A different gene, *afs*R, which encodes a positive regulatory protein for production of A-factor and several pigments, was cloned from *S. coelicolor* [42]. This gene, when introduced into an A-factor-deficient mutant strain of *S. lividans*, restored A-factor formation and led to production of a large quantity of the pigments actinorhodin and undecylprodigiosin, which are normally expressed at very low levels in *S. lividans* [41,42]. A cascade of expression initiated by the protein product of the *afs*R gene has been proposed to explain control of secondary metabolism and differentiation in *Streptomyces* spp. [41]. *afs*R stimulates transcription of the actinorhodin pathway [44].

Although the cloned *afs*R was assumed to coincide with the *afs*B gene, subsequent experiments have shown that the *afs*R product is a bypass function with regard to *afs*B complementation [86]. Nucleotide sequencing of the *afs*R gene indicated that it codes for a 993-amino acid protein (M_r 105 600) which contains A- and B-type ATP-binding consensus sequences at its NH_2-terminal portion and two DNA-binding consensus sequences with a helix-turn-helix motif at its carboxyl-terminal region [43].

The purified *afs*R protein was found to be phosphorylated through the transfer of the τ-phosphate group of ATP in the presence of the cell extracts of *S. coelicolor* A3(2) and *S. lividans* [34]. The extent of phosphorylation of the *afs*R protein may modulate its regulatory activity. This finding is of great interest in the context of the well-known mechanism of the phosphate control of the biosynthesis of antibiotics and other secondary metabolites [59].

A different gene, *saf* (for '*s*ecretion-*a*ctivating *f*actor'), cloned from *S. griseus*, is involved in a common control mechanism for at least five extracellular enzymes, pigment formation, and differentiation [66]. The deduced amino acid sequence indicates that the *saf* gene product interacts with DNA. A DNA-binding domain typical of several regulatory proteins is present in *saf* [21] and *afs*R [41] polypeptides.

It is very likely that these DNA-binding polypeptides interact specifically with regulatory sequences of genes involved in secondary metabolism. Alternatively, these polypeptides may control expression of genes involved in the formation of A-factor and related pleiotropic effectors that in turn control expression of clusters of genes encoding enzymes of secondary metabolism.

The potential usefulness of widely acting regulatory systems is still unclear. The *saf* gene is present with different degrees of homology in most (if not all) *Streptomyces* tested [21]. The lack of specificity of these widely acting systems may lead, when they are amplified, to overexpression of other heterologous pathways. However, not enough information is available, at present, to substantiate this claim.

When a large segment of DNA encoding a biosynthetic pathway is cloned there is good chance of carrying a pathway-specific regulatory gene. The cluster of genes may be introduced into the wild type to amplify the entire cluster. Alternatively, random cloning of small fragments (2–5 kb) from primary clones into high-copy (or low-copy) number plasmids into the wild type may well lead to overproduction of the antibiotic due to the increased formation in the cell of positive effector molecules.

FUTURE OUTLOOK

Expression of antibiotic biosynthetic genes which are organized in clusters takes place frequently in a coordinated form. The availability of the clusters of genes will facilitate the study of the molecular mechanisms that control gene expression during antibiotic biosynthesis. Characterization of *cis*-acting DNA sequences and *trans*-

-acting regulatory genes is of great interest to understand the conditions that allow gene expression and to optimize antibiotic biosynthesis by removal of bottlenecks in the pathway.

Cloning techniques are ideally suited to achieve overexpression of pathway-specific positive regulatory genes. In those cases that may be controlled by negatively acting regulatory effectors, inactivation of the genes enclosing those effectors may lead to overexpression of the clusters. These strategies may be used to increase the yield of antibiotics of wild type strains, which are usually low producers. They may be also complementary to classical strain improvement by empirical mutagenesis.

The same strategies may be applied to achieve overproduction of secondary metabolites with different pharmacological activities, pigments, herbicides or plant growth regulators. Such products are similar in many respects to antibiotics except that they lack antimicrobial activity.

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