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β-Lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes

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A cosmid clone containing closely linked β-lactam antibiotic biosynthetic genes was isolated from a gene library of *Flavobacterium* sp. SC 12,154. The location within the cluster of the DNA thought to contain the gene for δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), the first step in the β-lactam antibiotic biosynthetic pathway, was identified by a novel method. This DNA facilitated the isolation, by cross-hybridization, of the corresponding DNA from *Streptomyces clavuligerus* ATCC 27064, *Penicillium chrysogenum* Oli13 and *Aspergillus nidulans* R153. Evidence was obtained which confirmed that the cross-hybridizing sequences contained the ACVS gene. In each case the ACVS gene was found to be closely linked to other β-lactam biosynthetic genes and constituted part of a gene cluster.

Key words: ACV synthetase/gene cluster/β-lactam antibiotics

Introduction

The isolation of genes involved in β-lactam biosynthesis is of considerable interest given the continued commercial and clinical importance of β-lactam antibiotics. In particular it is anticipated that the application of molecular genetic techniques to producers may lead to new approaches for strain improvement (Skatrud *et al.*, 1989) and the synthesis of the novel β-lactam structures.

It has been established that the biosynthethic pathways of penicillin and the cephalosporins/cephamycins are closely related and share the part of the pathway that leads to the formation of isopenicillin N (IPN) from L-α-aminoadipate, L-cysteine and L-valine (Nuesch *et al.*, 1987).

Rapid progress has recently been made in cloning β-lactam antibiotic biosynthetic genes from a variety of fungi and *Streptomyces* (Ingolia and Queener, 1989). The gene encoding IPN synthetase (IPNS), the second common enzyme of β-lactam biosynthesis, has been isolated from a number of producers (Samson *et al.*, 1985; Carr *et al.*, 1986; Ramon *et al.*, 1987; Leskiw *et al.*, 1988). DNA sequencing has shown that close homology exists between the IPNS genes (Weigel *et al.*, 1988) to an extent that allows their cross-hybridization (Shiffman *et al.*, 1988). It is therefore probable that the genes encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), the first step in the biosynthetic pathway, are also closely related. The isolation of an ACVS gene has not been reported to date.

Antibiotic biosynthetic genes are often clustered in *Streptomyces* species (Malpartida and Hopwood, 1984; Chater and Bruton, 1985), including those for β-lactam biosynthesis (Chen *et al.*, 1988). It has been established that linked genes involved in β-lactam (cephamycin C) biosynthesis are present in *Streptomyces clavuligerus* (Burnham *et al.*, 1987). This gene cluster was shown to contain the IPN epimerase, deacetoxycephalosporin C synthetase (DAOCS) and O-carbamoyl deacetylcephalosporin C hydroxylase (OCDAC-hydroxylase) genes. Kovacevic *et al.* (1989) also reported the cloning of the DAOCS gene from *S.clavuligerus* and obtained evidence suggesting that it was linked to the IPNS gene.

We have used the known tendency for antibiotic biosynthetic genes in prokaryotes to be closely linked and the common steps of the penicillin and cephalosporin biosynthetic pathways as a means to clone, by cross-hybridization, ACVS genes from widely diverged β-lactam producers.

Results

Isolation of the Flavobacterium sp. SC 12,154 ACVS gene

A 3.0 kb *Bam*HI DNA fragment from pBROC137 containing the *S.clavuligerus* ATCC 27064 DAOCS gene (Burnham *et al.*, 1987) was subcloned into the *Streptomyces* transformation vector pIJ702 (Katz *et al.*, 1983). The orientation of the cloned fragment allowed the isolation of a 2.1 kb *Kpn*I–*Pst*I fragment consisting of 1.5 kb of *S. clavuligerus* ATCC 27064 DNA containing only the DAOCS gene and 0.6 kb of pIJ702. The fragment was used as a probe to screen a plasmid gene library of the cephalosporin producer *Flavobacterium* sp. SC 12,154 (Singh *et al.*, 1982). The filters from the colony hybridization were washed using a stringency of 2 × SSC, 0.1% SDS at 60°C (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate). The 6.3 kb *Bam*HI insert from the plasmid so isolated (pBROC143, Figure 3A), was subcloned into the *Bgl*II site of pIJ702 and the resulting construct (pBROC147) introduced by transformation into *Streptomyces lividans* 66, which does not produce penicillins or cephalosporins. Assays of cephalosporin biosynthetic enzyme activity in cell free extracts of the transformants showed that pBROC147 could confer IPN

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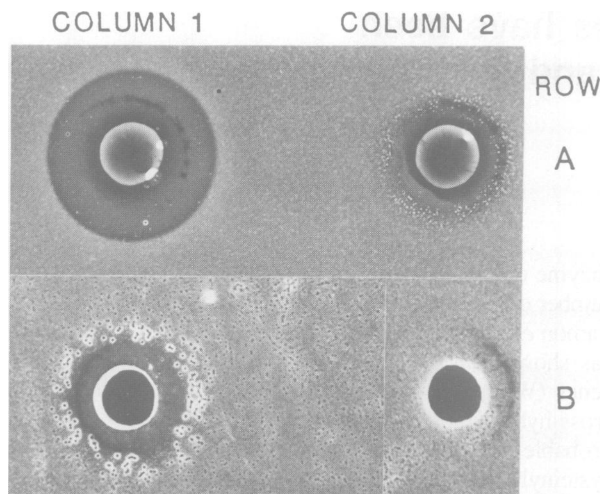

Fig. 1. Bioassay of IPN epimerase and DAOCS activity in cell free extracts of *S.lividans* 66 transformed with: **column 1**, pBROC147 containing *Flavobacterium* sp. SC 12,154 DNA; **column 2**; pIJ702 control DNA. **Row A**, IPN epimerase assay. Zone around well (**column 1**) represents inhibition of growth of *E.coli* ESS due to conversion of IPN to a more active antibiotic which was confirmed as deacetoxycephalosporin C by HPLC (see below). **Row B**, DAOCS assay. Zone around well (**column 1**) represents inhibition of growth of *A.faecalis* ATCC 8750 due to conversion of penicillin N to a penicillinase resistant antibiotic. HPLC analysis of the reaction products of both assays showed a peak eluting with a retention time identical to that produced by authentic deacetoxycephalosporin C (results not shown).

epimerase activity, as well as DAOCS activity, on *S.lividans* 66. Similar extracts prepared from control pIJ702 transformants did not possess any such activity (Figure 1).

A 0.9 kb *Bam*HI–*Xba*I DNA fragment containing most of the IPNS gene from *Penicillum chrysogenum* Oli13 (Smith *et al.*, 1989) was used to probe a Southern blot of total *Flavobacterium* sp. SC 12,154 DNA digested with *Bam*HI. The blot was washed using a stringency of 2 × SSC at 65°C and revealed a single hybridizing fragment identical in size to that cloned in pBROC143. Southern blots of restriction digests of pBROC143 DNA were probed under the same conditions with the *P.chrysogenum* Oli13 IPNS gene and the *Kpn*I–*Pst*I fragment containing the *S.clavuligerus* ATCC 27064 DAOCS gene. The *Flavobacterium* sp. SC 12,154 DNA in pBROC143 which hybridized to the probes is shown in Figure 3A. These experiments indicate the very close linkage of at least three cephalosporin biosynthetic genes in *Flavobacterium* sp. SC 12,154.

A *Flavobacterium* sp. SC 12,154 cosmid gene library was screened using the 6.3 kb *Bam*HI DNA insert of pBROC143 as a probe. Colonies containing hybridizing cosmid DNA were isolated after washing at a stringency of 0.1 × SSC at 65°C. Contiguous *Bam*HI fragments from one of the cosmid clones, pBROC155, were individually used as probes against Southern blots of total *P.chrysogenum* NRRL 1951 DNA washed at a stringency of 2 × SSC at 64°C.

As expected, the 6.3 kb *Bam*HI fragment of pBROC155 containing the *Flavobacterium* sp. SC 12,154 IPNS gene hybridized to *P.chrysogenum* NRRL 1951 DNA fragments of the predicted size for those containing the *P.chrysogenum* IPNS gene. Two additional pBROC155 *Bam*HI fragments (FGX1 and FGX2; Figure 3A) hybridized to *P.chrysogenum* NRRL 1951 DNA. The only *P.chrysogenum* β-lactam biosynthetic genes thought to be in common with *Flavobacterium* sp. SC 12,154 are those concerned with the biosynthesis of IPN. As the location of the *Flavobacterium* sp. SC 12,154 IPNS gene had already been identified it was possible to assign FGX1 and FGX2 as probably containing all or part of the gene encoding ACVS. No other DNA fragments of pBROC155 hybridized to total *P.chrysogenum* NRRL 1951 DNA at the stringency used.

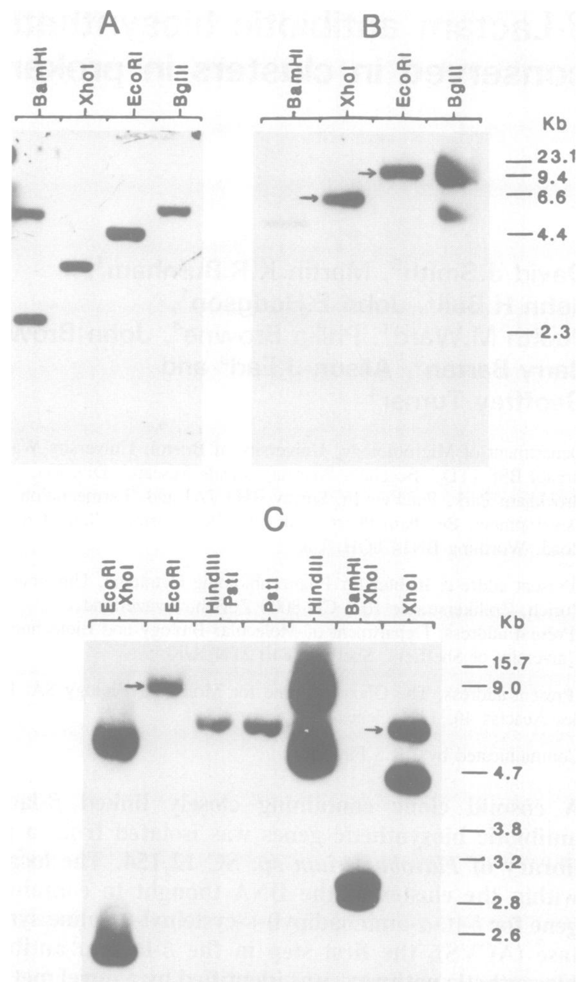

Fig. 2. Southern blot of restriction enzyme digests of *P.chrysogenum* Oli13 cosmid clone pCX3.2 DNA probed with: **A**, the *Bam*HI–*Xba*I fragment containing most of the IPNS gene of *P.chrysogenum* Oli13. Washing stringency was 0.2 × SSC, 0.1% SDS at 65°C; **B**, the *Flavobacterium* sp. SC 12,154 fragment FGX1. Washing stringency was 1.5 × SSC at 60°C; **C**, the *Flavobacterium* sp. SC 12,154 fragment FGX2, washing stringency as in **B**. The common 8.0 kb *Eco*RI and 5.5 kb *Xho*I bands in **B** and **C** are arrowed.

Identification of the S.clavuligerus ATCC 27064 ACVS gene

The 1.2 kb *Bam*HI–*Kpn*I fragment from plasmid pBROC137 containing the DAOCS gene of *S.clavuligerus* ATCC 27064 was used as a probe to isolate overlapping clones from an *S.clavuligerus* ATCC 27064 cosmid library. Hybridizing cosmid DNA was restriction mapped and a Southern blot of restriction digests of the clones probed with an 0.9 kb *Bam*HI–*Xba*I fragment containing the IPNS gene from *P.chrysogenum* Oli13 (washing stringency was 2 × SSC at 65°C). The probe hybridized only to DNA fragments from the region marked IPNS in Figure 3B. The *Flavobacterium* sp. SC 12,154 DNA fragments FGX1

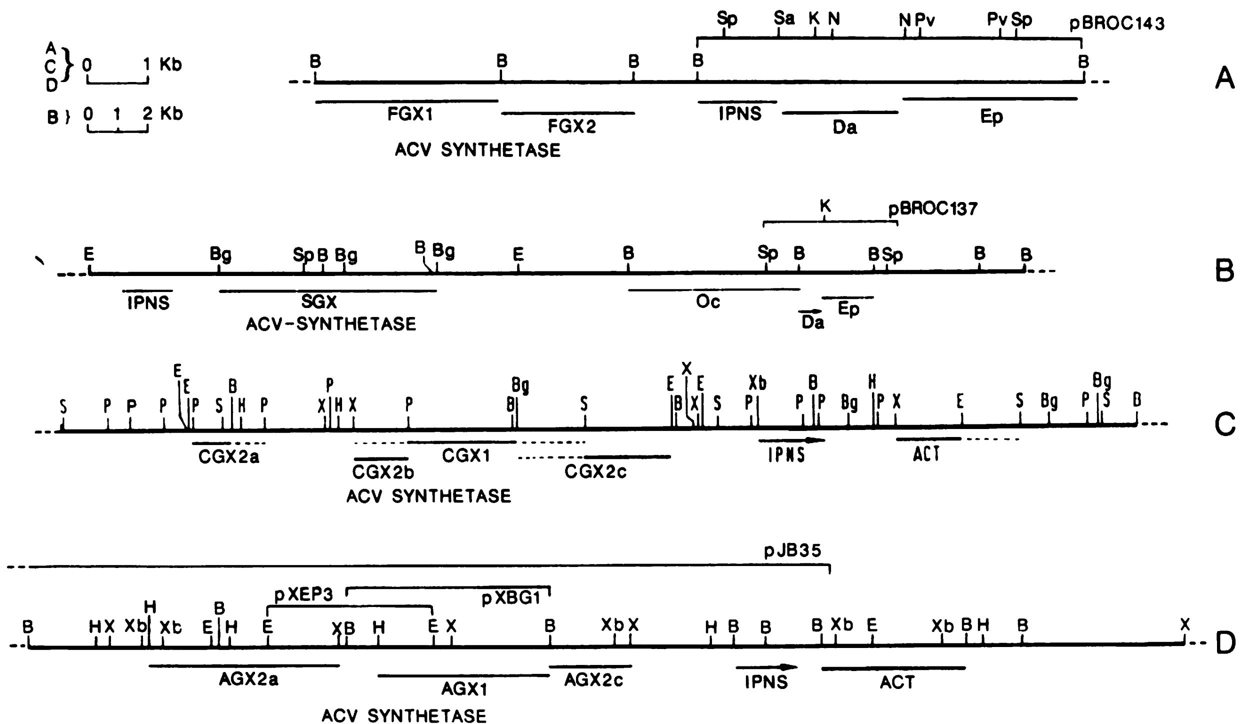

Fig. 3. Restriction and cross-hybridization maps showing, where appropriate, the approximate location of the genes encoding ACVS, IPNS, ACT, IPN epimerase (Ep), DAOCS (Da) and OCDAC-hydroxylase (Oc) in the β-lactam biosynthetic gene clusters from: **A**, *Flavobacterium* sp. SC 12,154; **B**, *S.clavuligerus* ATCC 27064; **C**, *P.chrysogenum* Oli13, FGX1 in **A** hybridizes to the region marked CGX1 and FGX2 to the regions marked CGX2 (a, b or c). Dotted lines indicate less strongly hybridizing regions. **D**, *A.nidulans* R153. The region marked AGX1 is that to which the CGX1 fragments from the equivalent *P.chrysogenum* Oli13 ACVS gene (C) hybridizes and the regions marked AGX2a and AGX2c are those which the *P.chrysogenum* Oli13 CGX2a and CGX2c fragments respectively hybridize. Arrows indicate direction of transcription where known. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; H, *Hin*dIII; X, *Xho*I; S, *Sal*I; K, *Kpn*I; N, *Nru*I; Sp, *Sph*I; Sa, *Sac*I; Pv, *Pvu*II; Bg, *Bgl*II; Xb, *Xba*I.

and FGX2, containing the putative ACVS gene, were also used as probes against restriction digests of the same *S. clavuligerus* ATCC 27064 cosmid clones. They hybridized at a similar stringency only to one region of the DNA termed SGX (Figure 3B). Southern blots of *S.clavuligerus* ATCC 27064 genomic DNA were similarly probed and showed fragments of the expected size to be present.

Isolation of the P.chrysogenum Oli13 ACVS gene

The FGX1 fragment of *Flavobacterium* sp. SC 12,154 was used as a probe to isolate hybridizing clones from a cosmid library of *P.chrysogenum* Oli13 screened using a washing stringency of 1.5 × SSC at 60°C. Restriction enzyme profiles of two of the hybridizing clones (pCX3.2 and pCX2.1) showed strong identity with pIPS4, a cosmid clone containing the IPNS gene isolated from the same gene library (Smith *et al.*, 1989).

Southern blots of restriction enzyme digests of pCX3.2 were probed with the 0.9 kb *Bam*HI–*Xba*I fragment containing most of the IPNS gene from *P.chrysogenum* Oli13 (Figure 2A). Hybridizing DNA fragments were detected that corresponded to the size expected for those containing the IPNS gene previously isolated on pIPS4. Similar restriction digests were also probed with the *Flavobacterium* sp. SC 12,154 FGX1 and FGX2 fragments (Figure 2B and C). Both probes hybridized strongly to separate fragments but an 8.0 kb *Eco*RI and a 5.5 kb *Xho*I fragment appeared to hybridize to both probes. Total *P.chrysogenum* Oli13 DNA was similarly probed which showed that fragments of similar size were present (results not shown).

A restriction enzyme map of part of pCX3.2 was constructed which showed that the region hybridizing to the FGX1 and FGX2 probes from *Flavobacterium* sp. SC 12,154 was located close to the IPNS gene previously isolated from pIPS4.

Southern blots of restriction digests of pCX3.2 were probed with the *Flavobacterium* sp. SC 12,154 fragments FGX1 and FGX2, allowing a cross-hybridization map to be constructed. The hybridization stringency was the same as that used in the screening of the gene bank. The FGX1 fragment hybridized only to the region of DNA in pCX3.2 marked CGX1 (Figure 3C) whereas FGX2 hybridized to three separate regions marked CGX2a, CGX2b and CGX2c. Comparison of the restriction enzyme patterns of cosmid clone pCX2.1 with pCX3.2 indicated that pCX2.1 also contained the CGX1 and CGX2 regions.

Identification of the Aspergillus nidulans ACVS gene

The CGX1 *Pst*I–*Bam*HI fragment from the putative ACVS gene in the *P.chrysogenum* Oli13 cosmid clone pCX3.2 (Figure 3C) was used as a probe to screen an *A.nidulans* R153 cosmid gene library using a washing stringency of 2 × SSC at 65°C. In this manner a clone termed pNGX1 was isolated which closely resembled cosmid pJB35 containing the IPNS gene of *A.nidulans* R153 (Brown, 1988). The CGX1, CGX2a and CGX2c fragments from the putative ACVS gene of *P.chrysogenum* Oli13 were used as probes against pNGX1 to construct a cross-hybridization map (Figure 3D) which showed the hybridizing regions to be located close to the IPNS gene.

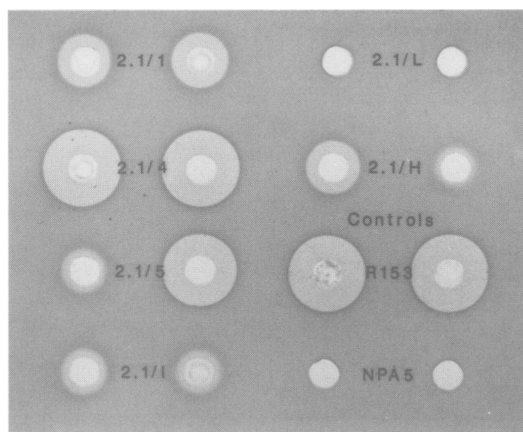

Fig. 4. Duplicate penicillin bioassays of six pCX2.1 transformants of *A.nidulans* NPA5 (2.1/1, 2.1/4, 2.1/5, 2.1/I, 2.1/L, 2.1/H), untransformed NPA5 and R153 (wild-type). Clear zones represent inhibition of growth of *Bacillus subtilis* ATTC 6633 due to a penicillinase sensitive antibiotic.

Identification of an additional fungal homologous sequence

Transformation experiments (see next section) suggested that sequences 3′ of the *A.nidulans* IPNS gene might be required for optimum restoration of penicillin production in a non-penicillin-producing (Npe$^-$) mutant of *A.nidulans*. An 8.0 kb *Xho*I fragment containing the *A.nidulans* R153 IPNS gene and sequences 3′ of it was used as a probe against a Southern blot of restriction enzyme digests of *P.chrysogenum* Oli13 total DNA and cosmid clone pCX3.2 (results not shown). The probe did not contain sequences extending into the previously identified regions 5′ of the IPNS gene containing the putative ACVS gene. The washing stringency was 0.5 × SSC at 65°C. As expected, *P.chrysogenum* Oli13 DNA fragments corresponding to the IPNS gene hybridized; however, other fragments (designated ACT) also having homology with the probe were located 3′ to the IPNS gene (Figure 3C). Diez *et al.* (1989) have reported that the acyl-CoA:6-aminopenicillanic acid (6-APA) acyltransferase (ACT) gene of *P.chrysogenum* is located close to the 3′ end of the IPNS gene. This corresponds to the hybridizing ACT region described above.

Complementation of the A.nidulans npeA0022 allele

Several complementation groups have been identified in *A.nidulans* Npe$^-$ mutants (Edwards *et al.*, 1974; Makins *et al.*, 1983). Mutants of the *npeA* group occur most frequently and are thought to be defective in ACVS activity (Makins *et al.*, 1981).

Penicillin bioassays were performed on *A.nidulans* NPA5 (bearing the *npeA002* allele) transformants obtained using the *P.chrysogenum* cosmid clone pCX2.1 (described above) and pPCY7. Plasmid pPCY7 was constructed by subcloning the *Xho*I fragment containing the IPNS gene of *P. chrysogenum* Oli13 into the *A.nidulans* transformation vector pDJB3 (Ballance and Turner, 1985). All 15 pPCY7 transformants tested and untransformed NPA5 did not produce any detectable antibiotic while five of the six pCX2.1 transformants tested produced an antibiotic that was sensitive to penicillinase (Figure 4).

Bioassays were performed on transformants of *A.nidulans* NPA5 obtained with the *A.nidulans* R153 cosmid clones pNGX1 and pJB35. All transformants produced a penicillinase sensitive antibiotic; however, those transformants obtained using pJB35 (which does not contain sequences extending far beyond the 3′ end of the IPNS gene, Figure 3D) produced less antibiotic than those obtained using pNGX1. This indicated that the DNA 3′ of the *A.nidulans* IPNS gene had a function affecting penicillin biosynthesis.

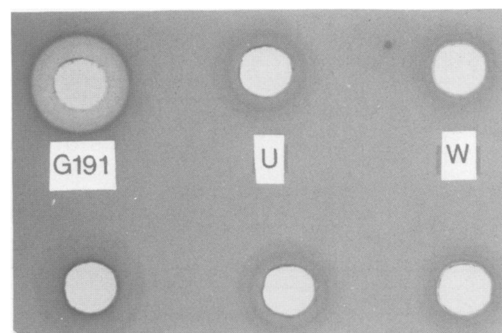

Fig. 5. Penicillin bioassays of *A.nidulans* G191 and pXBG1 transformants U and W. Bottom row treated with penicillinase.

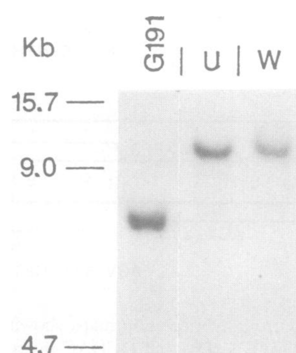

Fig. 6. Southern blot of *Xba*I digests of total DNA from *A.nidulans* G191 and pXBG1 transformants U and W. Probed with the 1.2 kb *Eco*RI–*Xho*I fragment from pXEP3 (Figure 3D). Washing stringency was 0.1 × SSC at 65°C.

Disruption of the A.nidulans ACVS gene

The putative *A.nidulans* ACVS gene was disrupted by transformation of penicillin-producing *A.nidulans* G191 with vectors containing internal ACVS gene DNA fragments.

Plasmid pXBG1 was constructed by subcloning the 3.2 kb *Bam*HI fragment indicated on Figure 3D into the *A.nidulans* transformation vector pGM32. An Npe$^-$ phenotype was obtained in six out of 10 transformants obtained using this plasmid and bioassays of two of these are shown in Figure 5. Southern analysis indicated, by an increase in *Xba*I band size from 7.5 kb to 11.5 kb when probed with the 1.2 kb *Eco*RI–*Xho*I fragment from pXEP3 (Figure 3D), that transforming DNA had integrated at the ACVS locus in both the Npe$^-$ transformants (Figure 6).

An Npe$^-$ phenotype was also obtained when pXEP3, constructed by subcloning the 2.8 kb *Eco*RI fragment shown in Figure 3D into the *A.nidulans* transformation vector pGT1, was used in similar experiments (results not shown).

Transcript analysis of the P.chrysogenum ACVS, IPNS and ACT genes

Northern blots of total RNA made from *P.chrysogenum* BW1890 (high penicillin-producing strain) and NRRL 1951

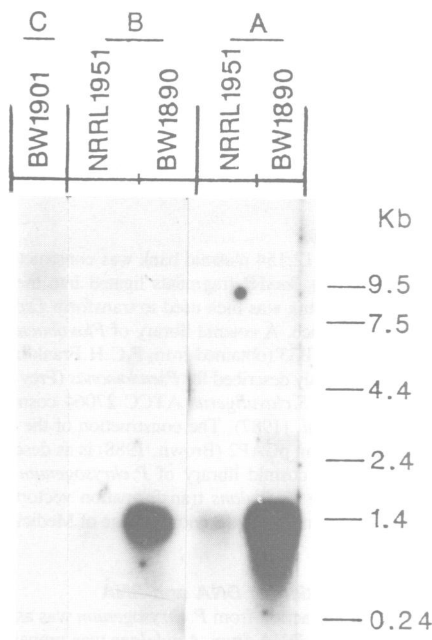

Fig. 7. Northern blot of total *P.chrysogenum* BW1890 and NRRL 1951 RNA prepared from 45 h mycelia probed with: **A**, the *Bam*HI–*Xba*I fragment containing the IPNS gene; **B**, the *Bam*HI–*Hin*dIII DNA fragment containing the ACT gene. BW1901 RNA probed with: **C**, a single-stranded RNA probe synthesized from the ACVS gene. 15 μg of RNA was applied in each lane.

(wild-type) were probed with the *P.chrysogenum* Oli13 IPNS and ACT genes. In addition RNA from *P.chrysogenum* BW1901 (high penicillin-producing strain) was probed with a single-stranded ACVS gene probe (Figure 7).

Strongly hybridizing bands at 1.4 kb were obtained with both the IPNS and ACT gene probes on BW1890 RNA (Figure 7). A band of similar size was detectable in the NRRL 1951 tracks. BW1901 RNA was probed with the ACVS gene and showed a single transcript >9.5 kb in size which was present at a very much lower level in NRRL 1951 RNA (not shown). The use of a single-stranded ACVS probe showed that the direction of transcription of this gene was opposite to that of the IPNS gene.

Discussion

Our initial studies showed that, judging from the close proximity of the IPNS, IPN epimerase and DAOCS genes on a cosmid clone of *Flavobacterium* sp. SC 12,154 DNA, β-lactam biosynthetic genes were very closely linked in this organism. It was also found that, whereas the *S.clavuligerus* and *P.chrysogenum* IPNS genes hybridized poorly to each other, the *Flavobacterium* sp. SC 12,154 IPNS gene hybridized at an easily detectable level to the equivalent gene in both species, probably due to the GC content being intermediate between the two (M.Burnham, unpublished data).

These findings suggested to us a novel strategy whereby the location of the DNA encoding ACVS in *Flavobacterium* sp. SC 12,154 might be located by cross-hybridization experiments against *P.chrysogenum* DNA.

The location of the putative *Flavobacterium* sp. SC 12,154 ACVS gene was identified allowing the isolation, by heterologous hybridization methods, of the corresponding gene from *S.clavuligerus* ATCC 27064, *P.chrysogenum* Oli13 and *A.nidulans* R153. In each case the putative gene encoding ACVS was found to be linked to other β-lactam biosynthetic genes, so constituting a gene cluster.

We have previously demonstrated, by heterologous expression in non-penicillin-producing fungi, that all essential penicillin biosynthetic genes, including the ACVS, IPNS and ACT genes, are closely linked and contained on the *P. chrysogenum* Oli13 cosmid clone pCX3.2 (Smith *et al.*, 1990). That it is the cross-hybridizing regions close to the IPNS gene which contain the ACVS gene is deduced from the following observations.

In each case the ACVS gene was found to be closely linked to the IPNS gene, both of which are the only shared steps in the β-lactam biosynthetic pathways of *Flavobacterium* sp. SC 12,154, *P.chrysogenum*, *S.clavuligerus* and *A. nidulans*. In addition, the *P.chrysogenum* Oli13 ACVS gene (designated locus *acvA*) hybridized to DNA from the cephalosporin C producer *Cephalosporium acremonium* (D.Smith, unpublished data), but not to DNA from the related filamentous fungi *Neurospora crassa* and *Aspergillus niger*, which do not produce β-lactam antibiotics (Smith *et al.*, 1990).

Heterologous expression of a gene present in the *P. chrysogenum* Oli13 cosmid clone pCX2.1 could complement the *npeA0022* allele of *A.nidulans* NPA5 which is thought to be defective in ACV biosynthesis (Makins *et al.*, 1981). The IPNS gene from *P.chrysogenum* Oli13 could not complement NPA5 whereas an *A.nidulans* R153 cosmid clone which contained the ACVS gene (designated locus *acvA*) and the IPNS gene but lacked the ACT gene, could.

Disruption of the *A.nidulans* G191 *acvA* gene by the integration of a transforming plasmid at the *acvA* locus resulted in transformants with an Npe^- phenotype. The genes encoding IPNS and ACT remained intact in these strains.

The homology between the *acvA* genes of *A.nidulans* R153 and *P.chrysogenum* Oli13 extends for at least 8.0 kb, which is adequate to encode a large polypeptide of ~230 kd such as that shown to possess ACVS activity in *A.nidulans* (van Liempt *et al.*, 1989). An mRNA transcript from *P.chrysogenum* BW1901 >9.5 kb in size could be detected using a probe corresponding to the *P.chrysogenum* Oli13 *acvA* gene. The presumed direction of transcription of the *P.chrysogenum acvA* gene (opposite to that of the IPNS gene) is consistent with the identification by DNA sequencing of a long open reading frame in this direction (D.Smith, in preparation).

As has previously been demonstrated for the IPNS gene (Smith *et al.*, 1989), high levels of ACT and *acvA* mRNA were detected in high penicillin-producing strains of *P. chrysogenum* relative to wild-type.

The gene encoding ACT, which performs the last step in the penicillin biosynthetic pathway, has been cloned from *P.chrysogenum* (Diez *et al.*, 1989) and is closely linked to the IPNS gene. This corresponds to the ACT region identified in *P.chrysogenum* Oli13 by its homology with *A.nidulans* R153. The *A.nidulans* ACT gene has been located in a similar position relative to the IPNS gene and the locus designated *acyA* (J.Kinghorn, personal communication).

The presence of antibiotic biosynthetic gene clusters in *Streptomyces* species is well documented (Malpartida

and Hopwood, 1984; Chater and Bruton, 1985). The identification of a β-lactam biosynthetic gene cluster in filamentous fungi contradicts a popularly held belief that, in these organisms, the genes are not linked (Makins *et al.*, 1983; Ramos *et al.*, 1986; Kovacevic *et al.*, 1988) although other gene clusters have been identified in filamentous fungi (Gurr *et al.*, 1987).

Skatrud and Queener (1989) have reported that the IPNS and DAOCS genes of *C.acremonium* reside on different chromosomes, indicating that not all cephalosporin C biosynthetic genes are linked in this organsm. It will be interesting to determine whether the gene encoding ACVS in *C.acremonium* is as closely linked to the IPNS gene as it is in the four species described here.

The results show that some of the *A.nidulans npe* and *pen* (penicillin titre increasing) mutations, which were mapped on different linkage groups (Makins *et al.*, 1983), cannot be in the structural genes of the penicillin biosynthetic pathway. The *npeA0022* allele used in this work has, however, undergone DNA rearrangements in the region corresponding to the *acvA* gene (J.Bull, unpublished data).

Of the five *P.chrysogenum* Npe^- complementation groups isolated by Normansell *et al.* (1979), three were shown to be on the same arm of one linkage group (Saunders and Holt, 1987), indicating that these may be genuine mutants in the penicillin biosynthetic gene cluster.

The significance of clustering of functionally related, but separately transcribed, genes in eukaryotes is not clear although it may reflect a common mechanism of regulation. In the case of β-lactam biosynthetic genes it is perhaps more likely that it reflects a common ancestral origin.

It has been suggested (Ramon *et al.*, 1987; Weigel *et al.*, 1988) that β-lactam biosynthetic genes may have been transferred from a prokaryote to a eukaryotic progenitor of those fungi which synthesize β-lactam antibiotics. The presence of β-lactam biosynthetic gene clusters in Gram-negative and Gram-positive prokaryotes, which are conserved in eukaryotes, supports the hypothesis that some of the biosynthetic genes were transferred as a cluster.

The *Flavobacterium* sp. SC 12,154 FGX2 probe hybridizes to three distinct regions of the *P.chrysogenum* Oli13 *acvA* gene. This may indicate that conserved domains within the ACVS protein have been rearranged between *Flavobacterium* sp. SC 12,154 and *P.chrysogenum* Oli13, or that ACVS activity is not encoded by a single gene in *Flavobacterium* sp. SC 12,154.

Smith *et al.* (1989) showed that the gene encoding IPNS was present in repeated copies in a high penicillin-producing strain of *P.chrysogenum*. The amplified region extended for >38 kb and our results show that it contains all the essential penicillin biosynthetic genes.

The identification of conserved gene clusters for β-lactam biosynthesis in prokaryotes and eukaryotes, containing the previously unidentified gene encoding ACVS, will assist the full elucidation of the pathways of β-lactam antibiotic biosynthesis and the factors governing their regulation.

Materials and methods

Strains and culture

The properties and conditions for culture of *Flavobacterium* sp. SC 12,154 are detailed in Singh *et al.* (1982). *S.clavuligerus* ATCC 27064 and *S.lividans* 66 were cultured using standard methods (Hopwood *et al.*, 1985). *P. chrysogenum* Oli13, NRRL 1951 and BW1890 are described in Smith *et al.* (1989). BW1901 was derived from BW1890 after mutagenesis and selection for increased penicillin titre. All *P.chrysogenum* strains were cultured on solid or liquid GM medium (Smith *et al.*, 1989). *A.nidulans* R153 (*wA3*; *pyroA4*), G191 (*pyrG89*, *pabaA1*; *uaY9*, *fwA1*), GH79 (*ya2*; *pyroA4*; *cnxA5*; *npeA0022*) and NPA5 (*fwA1*; *pyroA4*; *pyrG89*; *npeA0022*) were grown using standard methods (Ballance and Turner, 1985), *A.nidulans* GH79 was obtained from G.Saunders (Polytechnic of Central London) and *A.nidulans* NPA5 selected from a cross between GH79 and G191.

Gene bank construction

A *Flavobacterium* sp. SC 12,154 plasmid bank was constructed using size fractionated (5.0–7.0 kb) *Bam*HI fragments ligated into the *Bam*HI site of pAT153. The ligation mix was then used to transform *Escherichia coli* DH1 to ampicillin resistance. A cosmid library of *Flavobacterium* sp. SC 12,154 was made in pMMB33 (obtained from F.C.H.Franklin, University of Birmingham) as previously described for *Pseudomonas* (Frey *et al.*, 1983).

The construction of an *S.clavuligerus* ATCC 27064 cosmid library is described in Burnham *et al.* (1987). The construction of the cosmid bank of *A.nidulans* R153 DNA in pCAP2 (Brown, 1988) is as described for the construction of a pCAP2 cosmid library of *P.chrysogenum* Oli13 DNA (Smith *et al.*, 1989). The *A.nidulans* transformation vectors, pGT1 and pGM32, was obtained from G.May (Baylor College of Medicine, Houston, USA).

Isolation and manipulation of DNA and RNA

Total DNA and RNA extraction from *P.chrysogenum* was as described in Smith *et al.* (1989). Total DNA from *A.nidulans* was prepared using the method of Raeder and Broda (1985). The method used to isolate DNA from *S.clavuligerus* is given in Burnham *et al.* (1987).

Standard procedures were used for DNA and RNA manipulations, including small and large scale plasmid isolations, restriction enzyme digestion, ligation, *E.coli* transformation, colony hybridization, Southern and Northern blotting and DNA fragment isolation (Maniatis *et al.*, 1982). An Amersham Multiprime™ kit (Amersham International Plc) was used to prepare ^{32}P-labelled DNA probes. A single-stranded ACVS RNA probe was prepared as follows. The 8.0 kb *Eco*RI fragment containing most of the ACVS gene from *P.chrysogenum* Oli13 was cloned into the *Eco*RI site of pBluescript SK (Stratagene, La Jolla, USA) and ^{32}P-labelled RNA prepared by initiating transcription from the T3 promoter of the resulting vector according to the pBluescript instruction manual.

A.nidulans strains were transformed using the method of Ballance and Turner (1985). *S.lividans* 66 was transformed according to the method of Hopwood *et al.* (1985).

Antibiotic bioassay and enzyme assay

Penicillin bioassays were performed using standard techniques (Smith *et al.*, 1990).

Cell free, particulate free soluble enzyme preparations of *S.lividans* 66 transformants were prepared (Burnham *et al.*, 1987) for enzyme assays. IPN epimerase was assayed using the *E.coli* ESS bioassay system described by Jensen *et al.* (1983a) and DAOCS (ring expansion) assays were performed by bioassay (Kupka *et al.*, 1983) using *Alcaligenes faecalis* ATCC 8750 as cephalosporin sensitive strain (Claridge and Johnson, 1962). Deacetoxycephalosporin C was analysed by HPLC using previously published methods (Jensen *et al.*, 1983b).

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