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Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin

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Summary. The isopenicillin N synthetase (IPNS) gene has been isolated from wild-type *Penicillium chrysogenum* and used as a probe to detect the equivalent gene on Southern blots of genomic DNA from a mutant producing high levels of penicillin. The IPNS gene in this strain is contained within a region of DNA of wild-type restriction pattern that extends for at least 39 kb and is present at between 8 and 16 copies. The steady state level of IPNS mRNA in the mutant producing high levels of penicillin is between 32- and 64-fold of that of the wild type, suggesting that the rate of transcription of some or all of the copies has been increased. In addition we have also shown that both the IPNS mRNA and enzyme is present throughout the growth phase in both strains under the culture conditions used. IPNS enzyme activity is greatly increased in the strain with the high penicillin titre.

Key words: Gene amplification – Isopenicillin N synthetase – β-Lactams – *Penicillium chrysogenum*

Introduction

The genetic basis of penicillin overproduction in commercial strains of *Penicillium chrysogenum* is of considerable interest if molecular techniques are to be applied to future strain improvement. At present high titre strains are obtained by a programme of strain improvement through random mutagenesis and screening (Rowlands 1984). This approach has had considerable success in increasing the amount of penicillin obtained through fermentations of *P. chrysogenum*. Commercial strains now produce many hundreds of times more penicillin V than the progenitor of modern production strains, *P. chrysogenum* NRRL 1951 (Pirt 1987). If the genetic basis for this massive difference in titre can be identified then it may be possible to use a rational approach to increase titre levels further. Possible methods include increasing gene copy number, replacement of existing promoters with more efficient ones and removal or alteration of regulatory elements. A transformation system combined with the cloning of the genes involved in β-lactam biosynthesis are essential steps towards this goal.

Transformation systems for *P. chrysogenum* have recently been developed, and those that are based on a dominant selection procedure allowing production strains to be transformed should prove especially useful. These include use of the *Aspergillus nidulans amdS* gene (Beri and Turner 1987) to select for growth on acetamide as sole nitrogen source or utilising resistance to an antibiotic such as oligomycin (Bull et al. 1988) or phleomycin (Kolar et al. 1988). The biosynthetic pathway of the β-lactam antibiotics has been the subject of much investigation (for review see Nuesch et al. 1987). Despite this several steps in the pathway are not fully understood. However, the isopenicillin N synthetase enzyme has been purified from *P. chrysogenum* (Ramos et al. 1985). Isopenicillin N synthetase (IPNS) plays a central role in the β-lactam biosynthetic pathway as it catalyses the ring closure of the tripeptide δL-α-aminoadipyl-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N. The gene encoding the enzyme has been cloned from *Cephalosporium acremonium* (Samson et al. 1985), *P. chrysogenum* (Carr et al. 1986) and *A. nidulans* (Ramon et al. 1987).

Increasing the copy number of specific genes involved in β-lactam biosynthesis has been suggested as a way of increasing the penicillin titre of a β-lactam producing species. Skatrud et al. (1986) report that by transformation of *C. acremonium* with a vector containing the *C. acremonium* IPNS gene they were able to increase the titre of cephalosporin C above that of the parent strain. Several hundred transformants had to be screened in order to identify one with an increased titre. The basis of the increase in titre was not clear but could have been due to the integration of several copies of the gene into the genome in a position where they were efficiently expressed.

In this paper we report the detection of amplification of the IPNS gene in a strain of *P. chrysogenum* producing high levels of penicillin, as a result of a strain improvement programme. The change in IPNS mRNA level of this strain was monitored throughout the growth phase and compared with the wild type and the effect of these changes on the IPNS enzyme activity determined.

Materials and methods

Strains, media and growth conditions. P. chrysogenum NRRL 1951: wild type (Raper et al. 1944); HP60: a nicotinamide-requiring mutant of NRRL 1951; Oli13: an oligomycin-resistant mutant of HP60; BW 1890: a mutant yielding high levels of penicillin and derived from the Wisconsin series of *P. chrysogenum* strains by an industrial strain im-

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provement programme. *Escherichia coli* DH1 was used for construction of gene banks and propagation of plasmids.

Liquid medium for growth of *P. chrysogenum* was glycerol molasses (GM) medium made up of (in g/l): glycerol (7.5), molasses (7.5), yeast extract (5), NaCl (10), $MgSO_4 \cdot 7H_2O$ (0.5), KH_2PO_4 (0.006), $NH_4FeSO_4 \cdot 24H_2O$ (0.016), $CuSO_4 \cdot 7H_2O$ (0.0001), $CaSO_4$ (0.25). This was solidified with 1.5% agar where necessary. Incubation temperature was 25° C. Seed medium consisting of 35 g/l corn steep liquor, 15 g/l glucose, 5 g/l $CaCO_3$ and 8 g/l rape seed oil, pH 5.9, was used for growth of seed cultures for the fermenter, in which modified FM medium (lactose, 85 g/l; corn steep liquor, 35 g/l; phenoxyacetic acid, 6 g/l; $CaCO_3$, 10 g/l; $MgSO_4 \cdot 7H_2O$, 4.5 g/l; KH_2PO_4, 7 g/l; rape seed oil, 6 g/l; pH 6.0) was used. Fermenters were LH 500 series with 2 l pots, running at a 1.5 l working volume and fitted with Anglicon pH and temperature controllers. Fermentation conditions were: temperature 25° C, pH control between 6.6 and 6.8 using dilute H_2SO_4 and NH_4OH. Agitation was at 1200 rpm. Seed stage was in a shake flask for 48 h at 25° C with a spore inoculum of 10^5 spores/ml and a 10% cross from seed to final stage. Samples of 50 ml of culture were taken for RNA preparation for which the mycelium was filtered through muslin, washed twice with water, frozen in liquid nitrogen and freeze dried. Penicillin titres were assayed using standard methods (Brownlee et al. 1949).

Isolation and manipulation of DNA and RNA. Total DNA extraction from *P. chrysogenum* was by the method described for *A. nidulans* by Ballance et al. (1983). Total RNA was extracted from *P. chrysogenum* using the technique described for *A. nidulans* by Timberlake (1986). Northern blotting was performed on total RNA.

A cosmid gene library was constructed by ligating a size fractionated *Sau*3A partial restriction digest of total DNA isolated from *P. chrysogenum* Oli13 to the unique *Bam*HI site of the *A. nidulans* cloning vector pCAP2 (Turner and Ballance 1986), packaging the resulting molecules into lambda particles and transfecting *E. coli* DH1 to ampicillin resistance.

Standard procedures were used for DNA and RNA manipulations including small-scale plasmid isolation, restriction enzyme digestion, ligation, colony hybridisation, Southern and Northern blotting and DNA fragment isolation (Maniatis et al. 1982). A Bethesda Research Laboratories Hybri-Slot™ manifold was used to prepare slot blots. The DNA to be applied was first denatured by heating to 95° C for 5 min and the RNA by heating to 65° C for a similar length of time. Samples were applied in a volume of 100 µl of 10 mM TRIS-HCl, 1 mM EDTA, pH 7.5, and the slots washed through with 100 µl of the same buffer. Hybond N™ was used as the hybridisation membrane (Amersham International). DNA probes were labelled with ^{32}P using a nick-translation kit or random primer kit supplied by Amersham. Slot blots were exposed to preflashed Fuji RX film without screens at room temperature and scanned using a Joyce-Loebl Chromoscan 3 scanning densitometer.

IPNS assays. Cell-free extracts were prepared from NRRL 1951 and BW 1890 essentially as described by Ramos et al. (1985) with the following modifications. After sonication, extracts were centrifuged at $40000 \times g$ for 30 min. Aliquots of supernatant were then desalted on a PD-10 Sephadex™ column (Pharmacia Ltd.) pre-equilibrated with extraction buffer. The eluent fractions containing protein were concentrated using a Centricon-10 (10000 mol.wt. cut-off filter; Amicon Ltd., Glos., UK) and centrifuged at $6000 \times g$ for 45 min. The final concentration of protein was adjusted to 1 mg/ml for BW 1890 and 5 mg/ml for NRRL 1951.

The enzyme reaction was performed as described by Ramos et al. (1985) except that the incubation time was 5 min for BW 1890 and 30 min for NRRL 1951. In all cases control reaction mixtures were incubated containing (1) all reaction components and boiled cell extract (2) all reaction components and buffer and (3) extract and buffer. LLD-ACV dimer and pure samples of isopenicillin N (IPN) were kindly supplied by Beecham Pharmaceuticals Research Division. The reaction was stopped with methanol and triplicate samples applied to wells 11 mm in diameter in Nunc bioassay plates containing 200 ml of agar. For measurement of IPN produced by BW 1890, nutrient agar containing 0.1% 2,3,5-triphenyltetrazolium chloride and inoculated with *Bacillus subtilis* ATCC 6633 at a final concentration of 1×10^6 spores/ml was used. Standards of IPN of between 10 and 200 µg/ml similarly diluted with methanol were included. Plates were incubated at 37° C for 18 h. For measurement of IPN produced by NRRL 1951, tryptic soy agar inoculated with *Bacillus calidolactis* C953 at a concentration of 0.5×10^7 spores/ml was used. Standards of IPN of between 0.1 and 10 µg/ml were included and plates incubated at 46° C for 20 h. Good correlations were obtained in all bioassays between the diameter of the inhibition zones and the logarithm of IPN concentration. In all cases controls gave no inhibitory zones.

Results

Cloning of the IPNS gene

A cosmid gene library of *P. chrysogenum* Oli13 was constructed in the *A. nidulans* cosmid vector pCAP2. This vector contains the *Neurospora crassa pyr-4* gene, a sequence termed *ans1* that enhances transformation frequency in *A. nidulans* (Ballance and Turner 1985), a lambda *cos* site and a single *Bam*HI restriction site into which *Sau*3A partial digests of genomic DNA can be ligated. This library was screened by colony hybridisation using a heterologous oligonucleotide corresponding to nucleotides 205–265 of the *C. acremonium* IPNS gene-coding sequence (Samson et al. 1985) as a probe. Hybridisations were performed overnight in 6 × SSC at 55° C followed by 3 × 1 h washes in 1 × SSC, 0.1 SDS at 55° C (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate).

Two positively hybridising colonies amongst 3500 screened were identified on duplicate filters and shown to contain overlapping cosmid clones as judged by restriction mapping. A single 3.2 kb *Xho*I band common to both these clones hybridised to the oligonucleotide on Southern blots of the cosmids. The DNA from this region of the gel was extracted and the resulting fragment inserted into the *Sal*I site of pUC9 to give pCYX4 (Fig. 1). Sequence analysis of the cloned fragment identified it as the *P. chrysogenum* IPNS gene by comparison with the sequence of the IPNS gene from *P. chrysogenum* isolated by Carr et al. (1986). pCYX4 contains an intact IPNS gene plus over 2 kb of 5′ and 3′ flanking sequences.

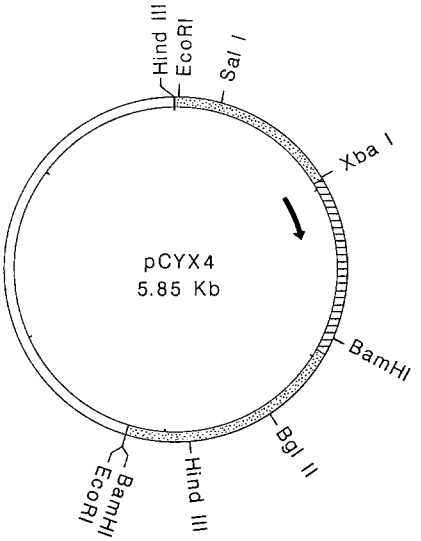

Fig. 1. Restriction map of pCYX4 containing the *Penicillium chrysogenum* isopenicillin N synthetase (IPNS) gene. The *open segment* indicates vector sequences, the *dotted segments* indicate *P. chrysogenum* DNA and the *cross-hatched segment* indicates the region encoding the *P. chrysogenum* IPNS. The *arrow* indicates the direction of transcription

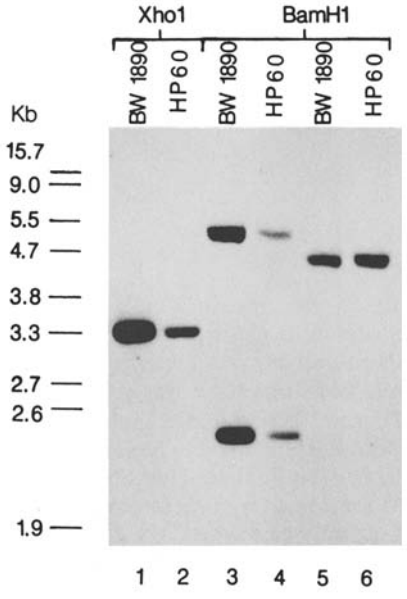

Fig. 2. Southern blot of *P. chrysogenum* DNA from strain BW 1890 (tracks 1, 3 and 5) and HP60 (tracks 2, 4 and 6). Five micrograms of DNA were loaded per track. Lanes 1–4 were probed with pCYX4 containing the *P. chrysogenum* IPNS gene and lanes 5 and 6 were probed with a plasmid containing the gene encoding an oligomycin-resistant subunit 9 protein of the *P. chrysogenum* ATP synthase (Bull et al. 1988)

DNA analysis

pCYX4 was used as a hybridisation probe against Southern blots of *Xho*I and *Bam*HI restriction digests of genomic DNA from BW 1890, a strain producing high levels of penicillin and HP60, a wild-type strain (Fig. 2). The intensity of the hybridising bands in tracks 1 and 3 of the BW 1890 DNA was found to be much greater than that of

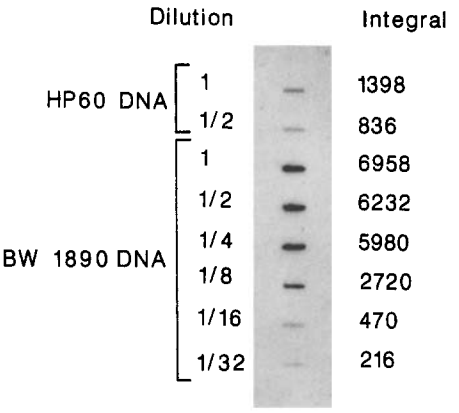

Fig. 3. Slot blot of DNA from HP60 and BW 1890 probed with pCYX4. Figures in the left-hand column refer to the dilution factor starting with 1 μg of DNA for both HP60 and BW 1890. Figures in the right-hand column refer to the integrals of the area under the peaks obtained from scanning densitometry of the autoradiograph and represent the band intensity in arbitrary units

strain HP60 tracks 2 and 4. Tracks 5 and 6 of this blot were probed with a cloned gene coding for the oligomycin-resistant subunit 9 protein of the *P. chrysogenum* ATP synthase (Bull et al. 1988). This gave a band of the same intensity for both BW 1890 and HP60 indicating, as expected, that this gene is present in equal and probably single copy in both strains.

To obtain an estimate of the number of copies of the IPNS gene in BW 1890, a slot blot containing 2-fold serial dilutions of a known quantity of BW 1890 DNA and a known quantity of HP60 DNA was prepared and probed with pCYX4 (Fig. 3). The autoradiograph was scanned using a densitometer. Comparison of the resulting integrals of the area under the peak, representing band intensity, of the successively diluted BW 1890 bands with the peak integrals of the HP60 bands, indicated that there were between 8 and 16 copies of the IPNS gene in BW 1890 for every 1 in HP60.

To determine the extent of the amplified region a Southern blot of BW 1890 and HP60 genomic DNA was probed with pIPS4, the cosmid clone from which the IPNS gene was isolated. This contains the IPNS gene and approximately 38 kb of flanking sequence (Fig. 4). All of the hybridising bands present in the HP60 DNA, tracks 2 and 4, were more intense in the corresponding BW 1890 DNA, tracks 1 and 3. The amplified region thus extends for at least 39 kb. It is not known whether this region is dispersed or tandemly repeated in the genome because the ends of the amplification were not detected.

RNA analysis

Samples of mycelium were taken from parallel fermentations of *P. chrysogenum* BW 1890 and NRRL 1951 grown in 1.5 l fermenters. Samples were collected at 16, 40, 65 and 90 h fermentation time after inoculation with a seed culture (see the Materials and methods). Total RNA was prepared from the eight mycelial samples and run on a denaturing gel which was blotted and probed with pCYX4 (Fig. 5). The RNA samples prepared from BW 1890 (lanes 1, 3, 5 and 7) contained a more intensely hybridising band than those containing NRRL 1951 RNA (lanes 2, 4, 6 and

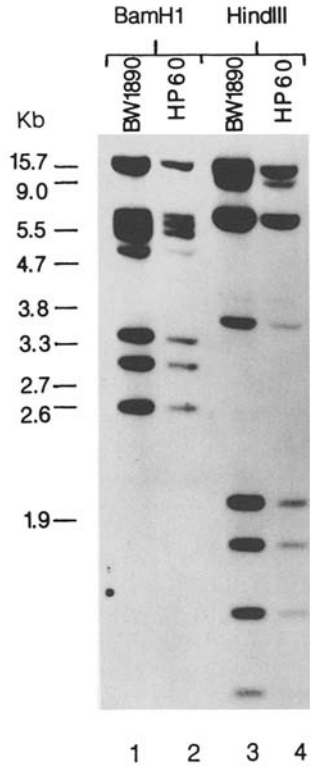

Fig. 4. Southern blot of *P. chrysogenum* DNA from strain BW 1890 (tracks 1 and 3) and HP60 (tracks 2 and 4) probed with pIPS4, a 47 kb cosmid containing the IPNS gene, 38 kb of flanking sequences and pCAP2 vector sequences. Five micrograms of DNA were loaded per track

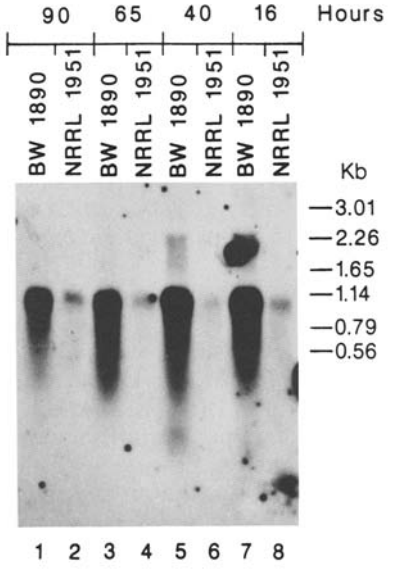

Fig. 5. Northern blot of total RNA isolated from NRRL 1951 (tracks 2, 4, 6 and 8) and BW 1890 (tracks 1, 3, 5 and 7) at 16, 40, 65 and 90 h. Fifteen micrograms of RNA were loaded per lane and the blot probed with pCYX4

8). A slot blot was performed as before on the RNA prepared from 16 h mycelia and probed with pCYX4. Following autoradiography, densitometer scanning and comparison of the integrals of the peak areas, representing band intensity, of the BW 1890 bands with the integrals of the

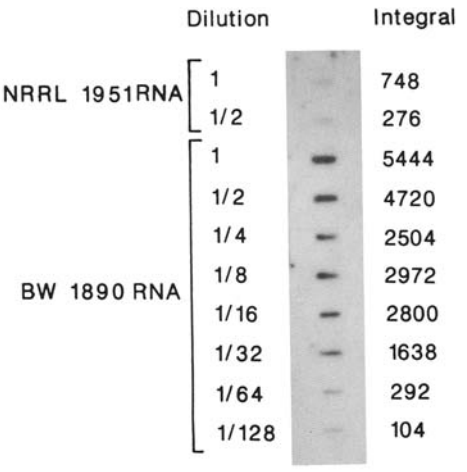

Fig. 6. Slot blot of NRRL 1951 and BW 1890 RNA prepared from 16 h mycelia and probed with pCYX4. Figures on the left refer to the dilution factor starting with 10 µg of applied material and figures on the right represent the band intensity in arbitrary units, as in Fig. 3

NRRL 1951 bands revealed that 16 h after inoculation IPNS mRNA was present in BW 1890 at levels of between 32- and 64-fold of that in NRRL 1951 (Fig. 6). This figure is considerably higher than the value obtained for the amount of DNA amplification.

IPNS mRNA was present in NRRL 1951 from 16 h through to 90 h fermentation time. Little variation in the level was observed over this period. In contrast the amount of BW 1890 IPNS mRNA appeared greatest at 16 and 40 h growth and had decreased slightly by 65 and 90 h (Fig. 5).

IPNS enzyme assays were performed on cell free extracts prepared from NRRL 1951 and BW 1890 mycelial samples obtained from similar fermentations to those from which the RNA samples were prepared. Samples were taken at 14, 38, 62 and 86 h after inoculation (Table 1). Very low IPNS activity was found in NRRL 1951 at all of these times, though the maximal level was measured at 38 h when penicillin began to accumulate in the medium. IPNS activity had decreased substantially by 62 and 86 h. The IPNS activity of BW 1890 was increased greatly over that of NRRL 1951. Maximal activity was again measured at 38 h inoculation but approximately half of the maximal level was present at 14 h growth when very little penicillin was detected in the medium. A high level of activity was maintained up to 86 h.

Penicillin assays were carried out on the fermenter samples from which the RNA was prepared (Fig. 7). Little penicillin was synthesised before 40 h, while it accumulated rapidly in both strains between 40 and 65 h. After 65 h no increase in titre was observed for NRRL 1951 but BW 1890 continued to accumulate penicillin at a slower rate. The amount of penicillin produced by BW 1890 was substantially greater than that from NRRL 1951.

Discussion

P. chrysogenum BW 1890 is the product of an industrial strain improvement programme and was derived from the Wisconsin strains of *P. chrysogenum* after extensive mutagenesis with a number of mutagenic agents. This strain im-

Table 1. Time course of isopenicillin N synthetase (IPNS) activity in parallel fermentations of NRRL 1951 and BW 1890. Activities are expressed as picomoles of IPN formed per min per milligram of protein. The variation given refers to the triplicate bioassays of the reaction products from a single enzyme assay at each time point (see the Materials and methods)

Strain	Time (h)			
	14	38	62	86
NRRL 1951	20.0± 0.5	89.7± 0.8	19.4± 0.3	15.2± 0.2
BW 1890	8260 ±220	15260 ±490	10080 ±140	13450 ±200

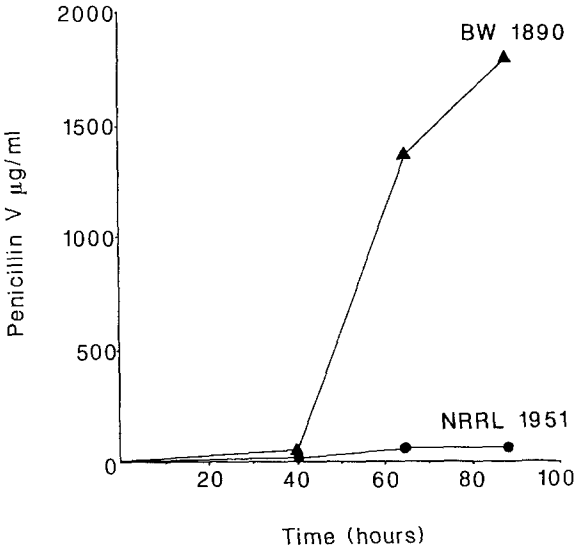

Fig. 7. Time course of penicillin V production from NRRL 1951 (●) and BW 1890 (▲). Single titre determinations were performed at each time point

provement programme has resulted in significant increases in gene copy number for at least one of the essential steps in the penicillin biosynthetic pathway. This is reflected in greatly increased mRNA and enzyme levels.

Each copy of the IPNS gene present in BW 1890 is contained within a region of DNA of wild-type restriction pattern that extends for at least 39 kb. This region is present at between 8 and 16 copies and appears to be maintained in a stable manner. Without detection of the junction fragments between the amplified region and the chromosome, it is not possible to elucidate whether the amplification is tandemly repeated or dispersed throughout the genome. The stability of an amplified gene in a filamentous fungus has been addressed by Wernars et al. (1985) who studied amplification of the *A. nidulans amdS* gene introduced into a corresponding *amdS* deletion strain by transformation. They found that the gene had integrated into the genome in tandem arrays, which had sometimes suffered rearrangements and which were inherited in a stable manner during mitosis but were not always meiotically stable.

The steady state level of IPNS mRNA in BW 1890 is between 32- and 64-fold of that of NRRL 1951 after 16 h growth. This indicates that the increase in message level is due to the increased number of gene copies plus an increased level of transcription of some or all of the copies above that observed in the wild type. This could be due to alterations in expression or activity of some *trans*-acting regulatory protein or by mutations to the 5′ regions of some or all of the genes resulting in increased promoter activity. The population of IPNS genes present in BW 1890 may be heterogeneous if promoter mutations occurred after the amplification step or between successive steps. The very high levels of IPNS enzyme detected in BW 1890 are probably due to a combination of these factors.

IPNS mRNA is present in both BW 1890 and NRRL 1951 in abundance at 16 h after inoculation with a seed culture. This correlates well with assays of IPNS enzyme activity, which show that 14 h after inoculation there is a detectable level of activity in NRRL 1951 while BW 1890 has approximately half of the maximal level. Ramos et al. (1985) also detected IPNS activity at 18 h growth in a strain of *P. chrysogenum* producing high levels of penicillin. Penicillin biosynthesis may take place at this early stage of growth but has not accumulated at a detectable concentration in the growth medium. Assays of penicillin concentration in the samples used to prepare the RNA showed that penicillin was present at a relatively low level at 40 h and increased rapidly between 40 and 65 h. This delay in the onset of penicillin biosynthesis, even though there is active IPNS enzyme present from a very early stage of the culture, may be necessary to allow amino acid levels to accumulate, in particular α-aminoadipic acid required for LLD-ACV synthesis (Jaklitsch et al. 1986). There is evidence that both α-aminoadipic acid and valine levels do increase internally in BW 1890 over this period (J. Edwards, unpublished data).

The quantity of IPNS mRNA remained at approximately the same level in NRRL 1951 from 16 h through to 90 h fermentation time. However, the level of IPNS mRNA present in BW 1890 appeared to be higher at 16 and 40 h than at 65 and 90 h. The large difference in IPNS mRNA levels between the two strains was maintained throughout the fermentation. This indicates that there is no strong regulatory control of IPNS at the transcriptional level under the growth conditions used to prepare samples for analysis, although both mRNA and enzyme may not have been assayed early enough in the final stage fermentation to detect an initial increase if it occurred.

The patterns of penicillin accretion correlated closely with the measurements of IPNS activity. In NRRL 1951 and BW 1890 a peak of IPNS was found at 38 h growth prior to the major increase in titre in which occurred after 40 h. High levels of enzyme were then maintained as penicillin accumulation continued. In NRRL 1951 a fall in enzyme activity by 62 h was accompanied by a cessation of penicillin production. However, BW 1890 maintained a high level of IPNS activity from 38 h up to 86 h, which was reflected

in the continuing accumulation of penicillin up to 90 h. This indicates that the regulation of penicillin biosynthesis in BW 1890 during the later stages of the fermentation may have been altered. The presence of IPNS mRNA in NRRL 1951 at 90 h suggests that this regulation is not due to IPNS transcription.

In order to study the regulation of the IPNS gene in *P. chrysogenum* further, a gene fusion of the 5′ region of the IPNS gene with the *E. coli* β-galactosidase gene has been constructed. This will be used together with direct RNA analysis to study the effect of various growth conditions on the regulation of the IPNS gene. This is the first reported instance of gene amplification resulting from strain improvement in a filamentous fungus.

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