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Overexpression of two penicillin structural genes in *Aspergillus nidulans*

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Abstract We have placed two different penicillin structural genes from *Aspergillus nidulans*, *ipnA* (encoding isopenicillin N synthetase, IPNS) and *acyA* (encoding acyl-CoA:6-aminopenicillanic acid acyltransferase, AAT), under the control of the strong *alcA* promoter [*alcA*(p)]. Single copies of these transcriptional fusions were targeted to the same chromosomal location and conditions have been worked out which simultaneously allow induction of the *alcA*(p) and support penicillin biosynthesis. Transcriptional induction of the chimeric genes *alcA*(p)::*ipnA* or *alcA*(p)::*acyA*(cdna) in the relevant recombinant strains results in 10-fold higher levels of the *ipnA* or *acyA* transcripts than those resulting from transcription of the corresponding endogenous genes. This increase causes a 40-fold rise in IPNS activity or a 8-fold rise in AAT activity. Despite this rise in enzyme levels, forced expression of the *ipnA* gene results in only a modest increase in levels of exported penicillin, whereas forced expression of the *acyA* gene reduces penicillin production, showing that neither of these enzymes is rate-limiting for penicillin biosynthesis in *A. nidulans*. A genomic version of the *alcA*(p)::*acyA* fusion, in which the *acyA* gene is interrupted by three small introns, is inducible by threonine to a lesser extent (as determined by both *acyA* mRNA levels and AAT enzyme levels) than the corresponding cDNA version, suggesting that processing of the introns present in the primary transcript may limit *acyA* expression.

Key words Filamentous fungi
Secondary metabolism
Alcohol dehydrogenase promoter
Isopenicillin N synthetase
Acyl-coenzyme A: isopenicillin N acyltransferase

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Introduction

Most natural isolates of *Aspergillus nidulans* synthesize penicillins (Holt and Macdonald 1968; Merrick and Caten 1975). Because *A. nidulans* is amenable to formal genetic analysis (Clutterbuck 1974) and to sophisticated molecular biology (Timberlake and Marshall 1989), this fungus represents a model organism for the study of the regulation of penicillin biosynthesis. Penicillin G biosynthesis from amino acids in *A. nidulans* (as well as in other *Plectomycetes*) takes place through the sequential action of three enzymes (reviewed in Luengo and Peñalva 1994): δ−(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthetase (IPNS) and acyl-CoA:6-aminopenicillanic acid acyltransferase (AAT). The genes encoding these enzymes, which are clustered on the right arm of chromosome VI (MacCabe et al. 1990), have been characterized. *ipnA* encodes IPNS (Ramón et al. 1987), *acyA* encodes AAT (Montenegro et al. 1990) and *acvA* encodes ACVS (MacCabe et al. 1991). In addition to these three enzymes, penicillin G biosynthesis requires active uptake of phenylacetate into the cell (Fernández-Cañón et al. 1989) and its conversion to phenylacetyl-CoA, one of the substrates of AAT, in a reaction probably mediated by general acyl-CoA synthetases (Martínez-Blanco et al. 1992).

The penicillin pathway of *A. nidulans* is regulated at least at the level of transcription of these structural genes (Peñalva et al. 1989; MacCabe et al. 1990). For example, levels of the *ipnA* mRNA (and penicillin biosynthesis itself) are under carbon source regulation (Espeso and Peñalva 1992; Pérez-Esteban et al. 1993). Also, inclusion in liquid minimal media of corn-steep liquor (CSL; Ligget and Koffler 1948), a complex additive rich in amino acids and peptides, results in simultaneous stimulation of penicillin biosynthesis and of transcription of the structural genes (MacCabe et al. 1990; Peñalva et al. 1992). However, even under optimal conditions for transcription of penicillin structural genes, *A. nidulans* secretes only modest amounts of penicillins. It

is possible that one or more catalytic steps in the pathway be rate-limiting, thereby modulating the overall synthesis of penicillin. The flux-control coefficent of individual enzymes in a pathway (a measure of the extent to which a given enzyme controls the flux through a metabolic pathway) is usually small [see Kell and Westerhoff (1986) for a review]. This might not be the case for the penicillin pathway because (i) pathways of secondary metabolism evolve under evolutionary pressures very different to those that shape primary metabolism, and (ii) it is likely that at least the gene encoding *ipnA* was horizontally transferred from bacteria (Peñalva et al. 1990). Non-optimal expression might have converted the IPNS-catalysed reaction to a rate-limiting step.

Recombinant DNA technology can be used to alter rate-controlling steps in biological pathways. For example, the level of a given enzyme can be changed by using a modulatable promoter to drive transcription of a gene, as shown by Walsh and Koshland (1985) for the *Escherichia coli* citrate synthetase. In this work, we use the strong *A. nidulans alcA* promoter, which can be regulated by the growth medium (Lockington et al. 1985; Gwynne et al. 1987; Waring et al. 1989), to drive transcription of the *ipnA* and *acyA* genes, encoding IPNS and AAT, respectively, and test whether the activity of either of these enzymes is limiting for extracellular penicillin accumulation.

Materials and methods

Aspergillus strains and media

A. nidulans biA1, metG1, argB2, veA1 was used as recipient strain in transformations. Recombinant strains constructed in this work are TRI [*biA1, metG1, argB2/argB*$^+$; *alcA*(p)::*ipnA*; *veA1*], TRA-gen [*biA1, metG1, argB2/argB*$^+$; *alcA*(p)::*acyA*(gen); *veA1*] TRA-cdna [*biA1, metG1, argB2/argB*$^+$; *alcA*(p)::*acyA*(cdna); *veA1*] and TRC [*biA1, metG1, argB2/argB*$^+$; *veA1*]. Strain maintenance, inoculation, harvesting, penicillin production broth, and bioassay for penicillins were as described previously (Espeso and Peñalva 1992; Pérez-Esteban et al. 1993). Variations in the corn-steep liquor (CSL) concentration introduced in particular experiments are discussed in the relevant sections.

Recombinant DNA and plasmid constructions

A 0.63 kb *Bgl*II-*Bam*HI fragment from pALC1 (Gwynne et al. 1987; Adams et al. 1988) containing a functional *alcA*(p) was subcloned in pBS-SK+ (Stratagene) in the *Sac*I to *Kpn*I orientation to yield pAP1. To construct the *alcA*(p)::*ipnA* fusion, pAP1 was cleaved with *Bam*HI and *Pst*I and mixed with the 194 bp *Bam*HI-*Nco*I fragment and the 1461 bp *Nco*I-*Nsi*I fragment of the *ipnA* gene (sequence in Ramón et al. 1987), and ligated to reconstruct the complete *ipnA* coding region and upstream sequences from position +49 relative to the major transcriptional start site (Pérez-Esteban et al. 1993). The *A. nidulans argB*$^+$ gene and the *trpC* transcriptional terminator (Mullaney et al. 1985) were then fused to this plasmid as a 3.2 kb *Eco*RI-*Kpn*I fragment (Pérez-Esteban et al. 1993), yielding pALIP. To construct the *alcA*(p)::*acyA*(gen) fusion, a 2096 bp *Ssp*I genomic fragment containing the complete coding region of the *A. nidulans acyA* gene (Montenegro et al. 1990) from position −29 relative to the start codon was ligated, downstream of the *alcA*(p), to *Sma*I-digested pAP1. The *argB*$^+$ gene was then inserted as a 3.2 kb genomic *Xba*I fragment into the single *Spe*I site, to yield pALAT-gen.

To construct the *alcA*(p)::*acyA*(cdna) fusion, we started from a cDNA clone isolated by Margarita Orejas from the random primed cDNA library described in Pérez-Esteban et al. (1993). This cDNA clone contains, inserted into the *Not*I site of pBS-SK$^+$, an incomplete copy of the processed *acyA* transcript starting at the 5′-untranslated leader (position −19 relative to the ATG) but lacking a few nucleotides at the C-terminal region of the ORF. To construct an intronless version of the *acyA* gene, a 0.64 kb *Sal*I (polylinker)-*Hpa*I fragment of this plasmid, which represents the spliced transcript lacking the three introns, was inserted into plasmid pCiAnH2 (Ramón et al. 1987) digested with *Sal*I and *Hpa*I. This reconstituted an intronless version of the *acyA* structural gene, which is followed by the genomic 3′-flanking region. Finally, the complete intronless gene plus the 3′-flanking region was excised from this construct as a 2 kb *Eco*RV-*Ssp*I fragment, which carries 45 bp of pBS-SK$^+$ polylinker sequences (which contain no ATG triplets), upstream of the 19 bp 5′-untranslated leader of the *acyA* cDNA used here. This fragment was inserted downstream of *alcA*(p) into *Sma*I-digested pAP1. The *argB*$^+$ gene was then inserted as a genomic 3.2 kb *Xba*I fragment into the single *Spe*I site, to yield pALT-cdna. Therefore, the 3′-flanking regions of the genomic and cDNA versions of the *alcA*(p)::*acyA* chimeras are identical, as both end at the *Ssp*I site. Finally, a control plasmid (pALC) carrying *argB*$^+$ was constructed by cloning the 3.2 kb *Eco*RI-*Kpn*I fragment mentioned above into pAP1. This places the *trpC* transcriptional terminator downstream *alcA*(p).

Aspergillus molecular biology

Transformation and identification of transformants carrying single-copy integrations of the different constructs at the *argB* locus were made as described previously (Gómez-Pardo and Peñalva 1990; Pérez-Esteban et al. 1993) using an *argB*-specific probe in Southern blots. RNA isolation from mycelia grown under penicillin-inducing conditions and Northern analysis as well as actin- and *ipnA*-specific probes have been described (Espeso and Peñalva 1992). The *acyA* probe was a 2 kb genomic *Bam*HI fragment (MacCabe et al. 1990), which contains the complete gene. Radioactive hybridization signals were quantified by using a PhosphorImager (Molecular Dynamics).

Assay of IPNS and AAT activities

Protein extracts used to measure enzyme activities were prepared as follows: mycelia were recoved by filtration, washed and suspended in 50 mM TRIS-HCl, pH 8.0, 0.1 mM DTT (for IPNS) or in 50 mM TRIS-HCl, pH 8.3 (for AAT). Cells were lysed by sonication in the cold, essentially as described by Ramos et al. (1985). Extracts were clarified by centrifugation (25000 × g, 15 min, 4°C). The supernatants, used in enzyme assays, usually contained a protein concentration of 5 mg/ml. IPNS activity was assayed essentially as described by Ramos et al. (1985), using 0.05 ml of the corresponding extract which was incubated at 25°C for the times indicated, in a 0.1 ml reaction volume containing 50 mM TRIS-HCl, pH 8.0, 0.2 mM δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (dimer form, Bachem, Switzerland), 0.1 mM SO_4Fe, 1 mM ascorbate and 2 mM DTT. Reactions were stopped after addition of an equal volume of methanol. Aliquots (0.1 ml) were used to estimate antibiotic activity in bioassays using penicillin G as standard. AAT activity was also measured (as phenylacetyl CoA: 6-APA acyltransferase) by bioassay as described in Luengo et al. (1986).

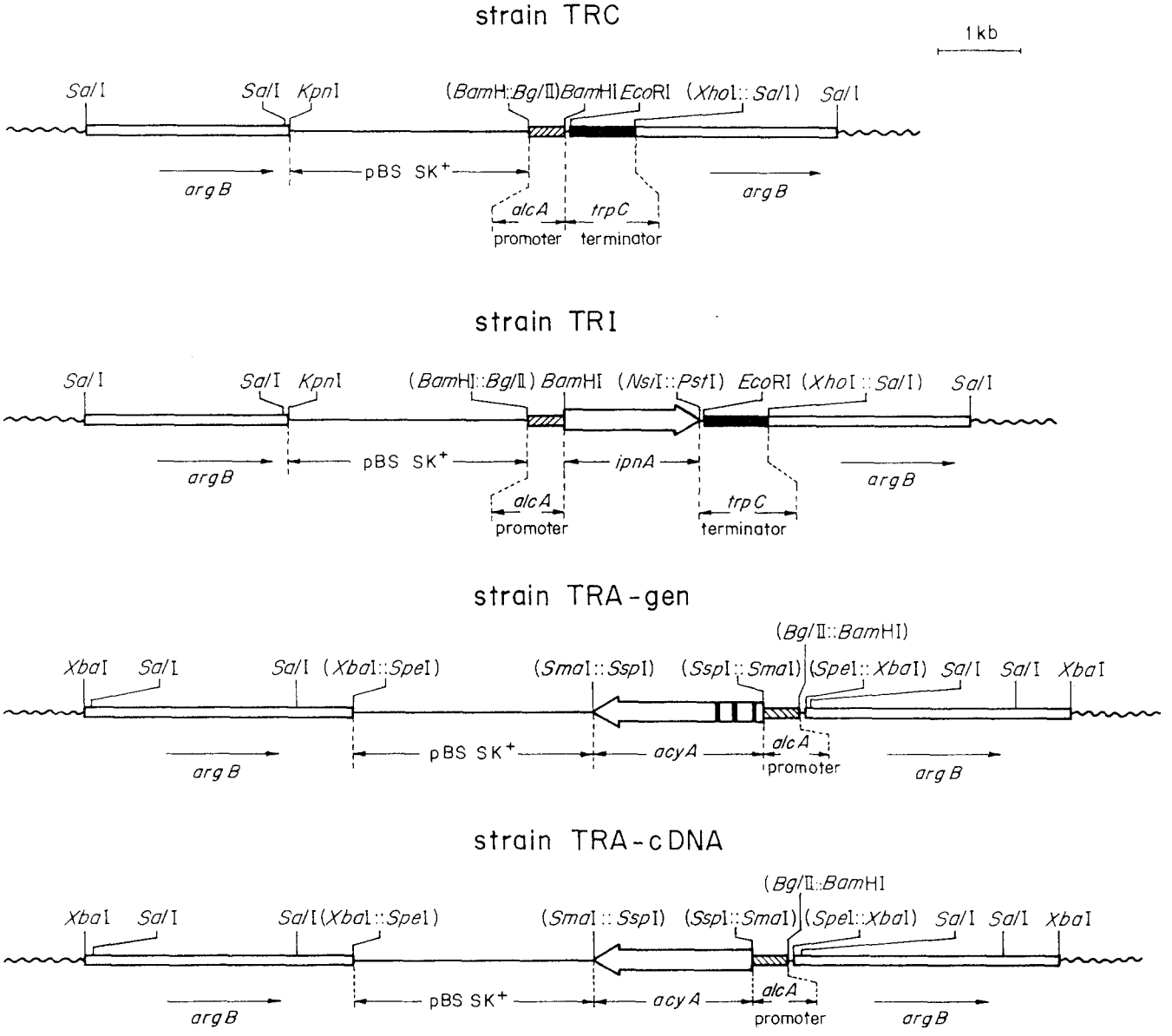


Fig. 1 Physical and genetic organization of single-copy integrations of the chimeric constructs at the *argB* locus in strains TRC, TRI, TRA-gen, and TRA-cdna. *Wavy lines* indicate chromosomal regions flanking *argB*. The fragment containing the *argB* gene is shown as *empty boxes*, pBS SK + sequences as *straight lines*, the *alcA* promoter as a *hatched box*, the *trpC* terminator as a *filled box* and the *ipnA* and *acyA* genes as *open arrows*. The positions of the three small introns in the *alcA*(p)::*acyA* gene carried by strain TRA-gen are indicated by *thick vertical lines*. Also indicated are some relevant restriction sites to facilitate understanding of plasmid construction strategies, described in Materials and methods

Results

Construction of strains carrying transcriptional fusions overexpressing the *ipnA* or the *acyA* transcripts

We have chosen the strong, well-characterised *A. nidulans alcA* gene promoter [*alcA*(p)] to drive expression of the *acyA* and *ipnA* genes. *alcA* (encoding alcohol dehydrogenase I) is controlled by glucose repression through the wide-domain transcriptional repressor CreA (Bailey and Arst 1975; Dowzer and Kelly 1991; Kulmburg et al. 1993) and is induced by alcohol through the pathway-specific transcriptional activator AlcR (Lockington et al. 1987; Kulmburg et al. 1991, 1992).

To construct the *alcA*(p)::*ipnA* fusion, the complete coding region of the *ipnA* gene, which is intronless (Ramón et al. 1987), was fused to the *alcA*(p) and the *trpC* transcriptional terminator, yielding plasmid pALIP (see Materials and methods). In contrast to *ipnA*, the *acyA* gene contains three introns (Montenegro et al. 1990). The putative lariat sequences of the first two introns diverge considerably from the consensus described in yeast (Parker et al. 1987). To test whether inefficient splicing might impair expression of AAT, we constructed two different *alcA*(p)::*acyA* expression plasmids, in which the *alcA*(p) was fused either to a genomic version of the *acyA* gene (plasmid pALAT-gen) or to a cDNA version (plasmid pALAT-cdna). No het-

erologous termination region was used in these two cases, as both constructs include a substantial portion of the *acyA* 3′-flanking region downstream of the *acyA* translation stop codon (see Materials and methods). Finally, a plasmid carrying the *alcA*(p) and the *trpC* terminator (containing no protein-coding region) was constructed as a control and named pALC. The four constructs (pALIP, pALAT-gen, pALAT-cdna and pALC) carried the *A. nidulans argB* wild-type gene in addition to the transcriptional fusions, and were used separately to transform an *A. nidulans argB*$^-$ strain to arginine prototrophy. Arginine-independent clones were purified and plasmid integrations were analysed by Southern blot hybridization, using *argB*-specific probes as described (Pérez-Esteban et al. 1993). Four different strains carrying single-copy integrations at the *argB* locus of pALIP, pALAT-gen, pALAT-cdna and pALC, respectively, were selected and named as follows: TRI (transformant overexpressing *ipnA*), TRA-gen (transformant overexpressing the *acyA* primary transcript), TRA-cdna (transformant overexpressing the spliced *acyA* transcript) and TRC (control). The locations of the chimeric fusions and the physical structure of DNA around the integration sites are shown in Fig. 1.

Design of the experimental system: expression of the *alcA* promoter under penicillin production conditions

Before testing the effect of the increased expression of the *acyA* and *ipnA* genes on penicillin biosynthesis, we first had to establish growth conditions which would lead, in complex penicillin production broth, to induction and derepression of the strong *alcA* promoter. Corn-steep liquor, which must be included in penicillin production broth to allow detectable penicillin acummulation by *A. nidulans* (Luengo and Peñalva 1994) has two opposing effects on the *alcA* promoter. At certain concentrations [above 1% (w/v), also see below] it causes *creA*-dependent carbon catabolite repression of *alcA*, as evidenced by prevention of lethality caused by toxic alcohols such as allyl alcohol in a *creA*$^+$ but not in a *creA*^{d}-2 background (data not shown). This *creA*^{d} allele is a strong loss-of-function mutation, which leads to almost complete derepression of *alcA* (Arst and Bailey 1977). Almost certainly, this effect is due to the high concentration of amino acids found in CSL, as a similar effect (although, predictably, at lower concentrations) was obtained with casamino acids. On the other hand, CSL contains threonine, a known inducer of *alcA* transcription (Lockington et al. 1985; Gwynne et al. 1987; Adams et al. 1988; Waring et al. 1989). Figure 2A shows that at starting concentrations of 0.5% glucose and 2.5% corn-steep liquor, *ipnA* transcript levels were similar in mycelia from the TRI and TRC strains after 24 h of growth. In contrast, *ipnA* transcript levels were strongly induced after 36 h of growth in strain TRI but not in strain TRC. Therefore, most of the high levels of the *ipnA* mRNA found in TRI were attributable to expression of the chimeric *alcA*(p)::*ipnA* fusion. This experiment confirmed the validity of the design of the transcriptional fusion. In addition, it showed that at 2.5% (w/v) CSL provides sufficient threonine to induce the *alcA* promoter. Finally, the *alcA* promoter is apparently repressed at 24 h, despite the fact that the glucose concentration has declined to non-repressing levels [substantially below 0.1% (w/v), data not shown]. Addition of exogenous threonine (100 mM final) at 9 h or 14 h after inoculation did not affect *alcA*(p) induction (not shown). Therefore, these data strongly suggest that CSL represses *alcA*(p) at 24 h, but not at 36 h, probably because at the later time the CSL has been partly catabolised following glucose depletion.

Late transcription of a penicillin-structural gene(s) might not result in higher penicillin levels, as the corresponding activity might be raised too late in the growth cycle to affect penicillin biosynthesis. Therefore, it was essential to advance the window of expression of the chimeric gene. It is possible to achieve this by simultaneously reducing initial concentrations of glucose and CSL. For example, in 0.1% glucose-broth, CSL concentrations of 0.5 or 1.5% did not give rise to *creA*-dependent *alcA* repression at 12 h after inoculation (see

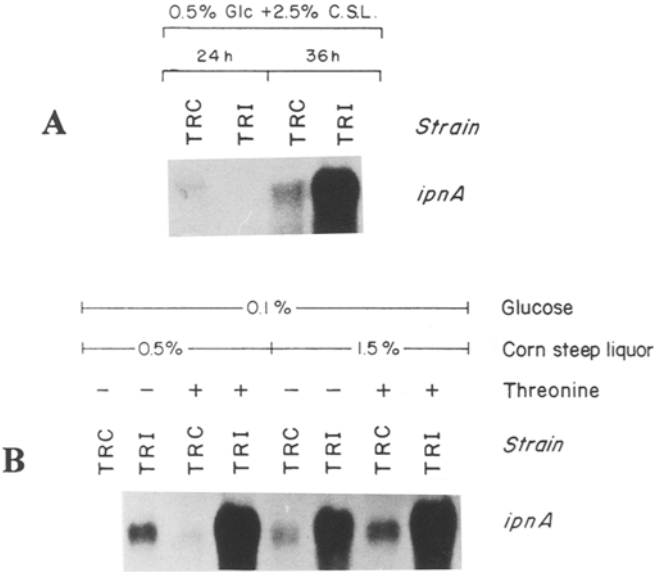


Fig. 2A, B Effect of corn steep liquor on *alcA*(p). Samples (10 μg) of total RNA isolated from mycelia of the indicated strains were analysed by Northern hybridization using an *ipnA*-specific probe. Loadings were aproximately equivalent, as deduced from intensity of rRNA bands. Culture conditions and the times after inoculation at which mycelia were harvested are shown in panel **A**. The cultures in panel **B** were grown for 12 h in penicillin production broth containing 0.1% (w/v) glucose and the indicated CSL concentrations. Inclusion or omission of 100 mM threonine in the broths is indicated by + and – symbols, respectively. The *ipnA* mRNA level corresponding to strain TRI, 24 h in panel **A** represents roughly half of the concentration detected in TRC (as shown by longer exposures). This difference is due to unequal loading and does not reflect true differences in transcript levels between the strains

Fig. 2B). Under these conditions, inclusion of 100 mM threonine improved *alcA*(p)::*ipnA* induction even in 1.5% CSL broth (Fig. 2B). However, 0.5% or 1.5% CSL broth supports penicillin production only very poorly. Addition of CSL to 1% (w/v) at 12 and 24 h after inoculation did not improve the final yield of antibiotic (data not shown). Therefore, we finally tested a broth containing 0.1% glucose, 2.5% CSL and 100 mM threonine, otherwise identical to that described previously (Espeso and Peñalva 1992). This broth supports penicillin biosynthesis and allows strong induction of *alcA*(p)::*ipnA* at 12 h after inoculation (see below), because the limiting initial glucose levels result in early CSL utilisation (thereby releasing catabolite repression caused by CSL). Therefore, this broth was chosen as standard for expression of *alcA*-based fusions.

Forced transcription of the *ipnA* and *acyA* genes in mycelia grown under conditions favourable for penicillin biosynthesis

Forced transcription of the *ipnA* and *acyA* genes was obtained by growing mycelia of strains TRI, TRA-gen and TRA-cdna in the broth mentioned above. Strain TRC (see Fig. 1) was grown under the same conditions as a control. Mycelial samples were taken at different times after inoculation and used to isolate RNA, which was analysed by Northern hybridization using *ipnA*- or *acyA*-specific probes. Figure 3 (left panel) shows that the *ipnA* mRNA levels were substantially higher in mycelia from the TRI strain (which contains the *alcA*(p)::*ipnA* fusion) than in mycelia from the control strain at both 12 h and 24 h after inoculation. The *ipnA* transcript levels corresponding to strain TRI (12 h sample) were 11 times higher than those of the control strain. The *ipnA* mRNA levels in the control strain (grown in penicillin production broth containing 0.1% glucose) correspond to carbon derepressed levels (Espeso and Peñalva 1992). These levels represent of the order of 0.1% of total mRNA, as measured by abundance of cDNA clones (Pérez-Esteban et al. 1993). Therefore, at 12 h, approximately 1% of the mRNA in strain TRI corresponds to *ipnA* message.

The *acyA* mRNA levels were also substantially higher in threonine-induced mycelia carrying either of the *alcA*(p)::*acyA* fusions than in mycelia from the control strain TRC (Fig. 3, right panel). However, while at the 12 h time the *acyA* RNA levels in strain TRA-cdna were 9 times higher than in the control, the *acyA* mRNA levels in strain TRA-gen were only 5 times higher than in TRC. These results indicate that the transcriptional efficiencies of the genomic and cDNA versions of the *alcA*(p)::*acyA* fusions are not equivalent.

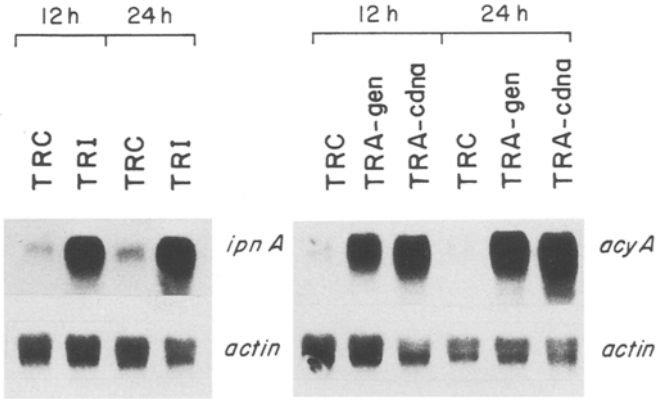
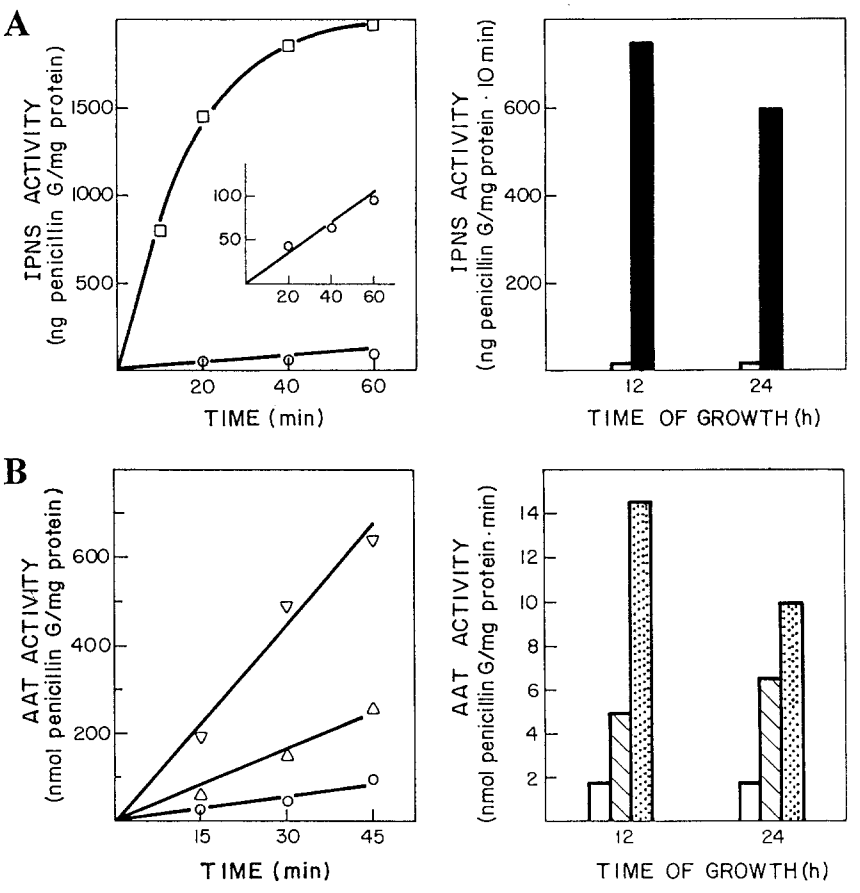


Fig. 3 Directed transcription of *ipnA* and *acyA* genes in strains TRI and TRA. Samples of total RNA (10 µg per lane) prepared from mycelia of the strains indicated grown at 37°C in penicillin production broth plus 100 mM threonine for 12 or 24 h, were analysed by Northern blot hybridization using *ipnA*- and *acyA*-specific probes. Blots were re-hybridised with an actin-specific probe to confirm equal loading

Enzyme activities in recombinant strains with increased levels of *ipnA* and *acyA* RNA

To test whether the elevated steady-state levels of *ipnA* or *acyA* mRNAs obtained in recombinant strains TRI, TRA-gen and TRA-cdna result in higher enzyme activities, protein extracts were made from the same mycelia used to measure transcript levels, and IPNS and AAT activities were assayed in vitro. The plots on the left side of Fig. 4 show the linearity of the assays for both IPNS (Fig. 4A) and AAT (Fig. 4B) activities in protein extracts from 12 h-old mycelia, in which the highest enzyme activities were found. The bar diagrams on the right show specific IPNS and AAT activities in the relevant recombinant strains compared to those in the control strain.

Overexpression of *ipnA* mRNA in strain TRI resulted in a 40-fold higher IPNS specific activity than in strain TRC (wild-type levels of *ipnA* and *acyA* RNAs). This remarkable elevation in IPNS activity obtained upon forced transcription is even higher than the increase that would be expected from the *alcA*(p)-driven rise in transcript levels (11 to 15-fold, Fig. 3). We have not investigated the reasons for this unexpectedly high enzyme accumulation. Forced transcription of *acyA* (genomic version) in strain TRA-gen resulted in a 3-fold higher AAT activity (12 h) than in the control TRC strain (see Fig. 4B). In contrast, overexpression of the cDNA version of *acyA* in strain TRA-cdna, resulted in an 8-fold increase in AAT activity in 12 h mycelia (Fig. 4B). The higher levels of AAT activity found in extracts from strain TRA-cdna relative to those found in extracts from strain TRA-gen correlate well with the differences in transcript levels, further supporting the conclusion that the *alcA*(p)::*acyA*-cdna chimeric fusion is more efficient than its genomic counterpart in terms of transcript accumulation. Although we cannot rule out differences in transcript stability, this would suggest that splicing of the non-canonical *acyA* introns might be rate-limiting for expression (see Discussion).



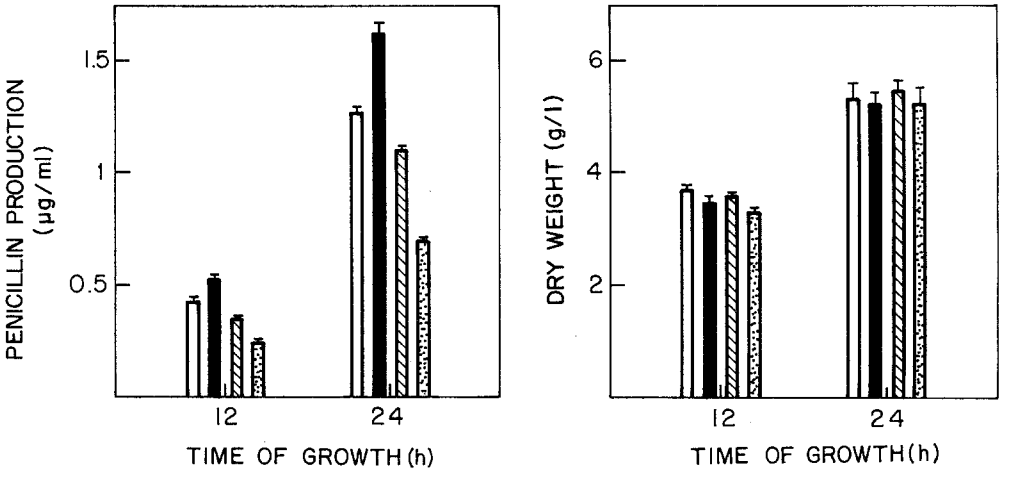


Fig. 4A, B Enzyme activities in recombinant strains. **A** Induction of IPNS activity in mycelial extracts from strain TRI. **B** Induction of AAT activity in mycelial extracts from strains TRA-gen and TRA-cdna. Time-courses on the left show the linearity of the rections used to assay isopenicillin N synthetase in strain TRI (□, panel **A**) and acyl-CoA: 6-aminopenicillanic acid acyltransferase (panel **B**) in strains TRA-gen (△) or TRA-cdna (▽). As a control, both activities were measured in strain TRC (○, panels **A** and **B**). Data correspond to the 12 h time, at which the highest enzyme activities were found. Bar diagrams on the right show IPNS activities (**A**) in extracts from strains TRI (*filled bars*) and TRC (*open bars*) and AAT activities (**B**) in extracts from TRA-cdna (*stippled bars*), TRA-gen (*hatched bars*) and TRC (*open bars*)

Penicillin biosynthesis by recombinant strains

Strain TRI contains a 40-fold higher activity of IPNS, and strains TRA-gen or TRA-cdna, respectively, contain a 3- or 8-fold higher activity of AAT than the control strain (at the 12 h time point). These strains allowed us to test whether either of these two enzyme activities limits penicillin biosynthesis. Penicillin production by cultures of strains TRI, TRA-gen, TRA-cdna and TRC grown at 37°C in penicillin production broth containing 0.1% glucose-2.5% CSL-100 mM threonine (conditions under which the four strains showed no differences in

Fig. 5 Penicillin production in cultures of recombinant strains. Flasks containing 0.1% glucose-2.5% corn steep-100 mM threonine penicillin production broth (which induces both penicillin production and expression of *alcA*(p) fusions) were inoculated with identical amounts of viable conidiospores of each different strain. Cultures were incubated at 37°C with shaking. At different times after inoculation, samples were taken to measure mycelial dry weight (right panel) and penicillin production (left panel). Data for each strain represent the average values obtained from three independent cultures. Under these controlled conditions, diffferences in growth between the different strains and flasks were almost negligible. Symbols are as follows: *open bars*, strain TRC; *filled bars*, strain TRI; *hatched bars*, strain TRA-gen and *stippled bars*, strain TRA-cdna

growth, Fig. 5, right panel) was measured at two different times in the growth cycle. Figure 5 (left panel) summarises data from three independent experiments. Elevated levels of IPNS (strain TRI) resulted in a modest, albeit reproducible increase in exported levels of penicillin (which were 25% higher than those obtained with the control strain TRC at 24 h; Fig. 5, left panel, filled and open bars). We conclude that IPNS activity does not limit penicillin biosynthesis, although a large increase in the amount of catalytic IPNS results in a slight rise in antibiotic production. In contrast, elevated levels of AAT activity (strains TRA-gen and TRA-cdna) reduced penicillin production (see Fig. 5, left). Therefore, we conclude that the amount of AAT activity is not rate-limiting for penicillin biosynthesis. The magnitude of the negative effect correlated with the extent to which AAT is overexpressed, being greater with the strain TRA-cdna (see Fig. 5, left), which produced roughly half of the penicillin levels made by the control strain at 24 h. Possible explanations for this effect will be discussed below.

Discussion

In *A. nidulans*, growth of the fungus on poor carbon sources (or on limiting levels of preferred carbon sources) results in higher penicillin production than in favourable carbon sources. This correlates with an at least 10-fold increase in *ipnA* mRNA levels (Espeso and Peñalva 1992). It has also been shown that AAT enzyme levels are reduced in glucose-grown cultures (Brakhage et al. 1992). However, it has never been tested whether the functions encoded by the *ipnA* and *acyA* genes are rate-limiting under favorable conditions for penicillin biosynthesis (i.e., carbon derepressing conditions). We address this issue by using a reverse genetics approach (Walsh and Koshland 1985) based on directed overtranscription of the corresponding structural genes.

Elevated transcription of the *ipnA* and *acyA* genes has been achieved using the *A. nidulans* alcohol dehydrogenase I gene (*alcA*) promoter, almost certainly the best characterised expression system available in filamentous ascomycetes (Waring et al. 1989 and references therein). This approach has previously been used succesfully in *A. nidulans*. Raising mRNA levels of regulatory genes involved in conidiphore development by using the *alcA*(p) has been crucial in elucidating their roles (Adams et al. 1988; Marshall and Timberlake 1991; Mirabito et al. 1989). However, the expression system based on *alcA*(p) had not been used with complex media such as those used to induce penicillin biosynthesis. As high levels of corn-steep liquor result in carbon catabolite repression of *alcA*(p), we describe here a broth composition which switches on the *alcA*(p) under conditions of penicillin production. Induction of the *alcA*(p)::*ipnA* and *alcA*(p)::*acyA*-cdna chimeric genes in this broth results in raising transcript levels by at least 10-fold over those of the corresponding endogenous genes. This rise in transcript levels causes an increase in the IPNS and AAT activities of 40- and 8-fold, respectively. The highest enzyme activities were achieved at 12 h after inoculation, i.e., well in advance of the time at which the highest penicillin levels were detected. Therefore, the growth and *alcA*(p) induction conditions used here were in principle valid for testing whether IPNS or AAT activities are limiting for penicillin production.

Overexpression of IPNS results in only a modest increase in penicillin biosynthesis, indicating that this enzyme is not rate limiting. In contrast, overexpression of AAT activity reduced penicillin biosynthesis. This proves that AAT activity is not limiting either. This negative effect of AAT overexpression is probably genuine, as it is also observed, although less prominently, in a strain which contains a genomic version of the *alcA*(p)::*acyA* fusion. This is consistent with the lower levels of AAT overexpression achieved with the genomic construct than with the cDNA construct. The negative influence on the synthesis of penicillins of the excess levels of AAT, relative to those of the other enzymes of the pathway may be mediated by the penicillin amidase (Alvarez et al. 1993) or phenylacetyl-CoA hydrolase (Martín Villacorta et al. 1991) activities of AAT.

Although neither of the enzymes limits penicillin biosynthesis to a susbstantial extent, in the case of AAT we cannot rule out the possibility that a large increase in its level results in incorrect subcellular localization of the excess enzyme (note that AAT is localized in microbodies, Müller et al. 1991). Also, we have not explored the possibility whether the increase in IPNS activity makes AAT limiting. In this context, Veenstra et al. (1991) reported clonal increments in penicillin production after transformation of a semi-industrial strain of *Penicillium chrysogenum* with a plasmid carrying the *P. chrysogenum* homologues of the *ipnA* and *acyA* genes. However, it is likely that in semi-industrial strains, the ratios of enzymes have been radically altered by selection, thereby changing the flux-control coefficients of individual enzymes. It is also worth noting that the *alcA*(p)::*acyA*(gen) chimeric gene gave rise to less transcript and lower enzyme levels than the *alcA*(p)::*acyA*(cdna) version. This suggests that processing of the *acyA* primary transcript may be inefficient, perhaps reflecting the divergence of the lariat sequences, not only from the 5′-TACTAAC-3′ yeast consensus (Parker et al. 1987), but also from the less stringent filamentous fungal consensus (Gurr et al. 1987).

The negligible or very small increase in penicillin levels obtained with the recombinant strains is not completely unexpected. The flux-control coefficient of most enzymes is small (Kell and Westerhoff 1986) and overexpression of a biosynthetic enzyme usually makes no difference to product formation (see Fraenkel 1992). In fact, many pathways have evolved with most of the flux-activity correlation focused on one enzyme. This is not the case for either of the two enzymes tested here. We have not yet tested the effect of overtranscription of the *acvA* gene, but ACVS, the enzyme encoded by this gene,

represents an obvious candidate for a rate-limiting step, as this huge multifunctional enzyme (MacCabe et al. 1991) catalyses the first steps in penicillin biosynthesis.

The finding that a particular production strain of *P. chrysogenum* contains an amplification of a 35 kb DNA region containing (at least) the penicillin gene cluster (Smith et al. 1989) can be rationalised in the context of our results. We show here that increasing the concentration of individual enzymes does not cause profound changes in the amounts of penicillin exported. The amplification in the production strain involves (at least) the three penicillin structural genes. It is very likely that simultaneous increases in the levels of enzymes encoded by this fragment result in a drastic change in pathway flux.

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