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δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*

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Abstract The *acvA* gene from *Aspergillus nidulans* encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase was overexpressed by replacing the wild-type *acvA* promoter with the ethanol dehydrogenase promoter, *alcAp*, from *A. nidulans*. The expression level of *alcAp* was determined using a strain in which the reporter gene, *lacZ*, is under the control of *alcAp*, and was found to be up to 100 times greater than that from the *acvA* promoter when induced in fermentation conditions. Penicillin yields were found to increase by as much as 30-fold when the *acvA* gene was overexpressed. Glucose, which strongly represses transcription from *alcAp*, also repressed penicillin biosynthesis in the overexpression strain. These results prove that ACV synthetase is a rate limiting enzyme for penicillin production in *A. nidulans*.

Key words ACVS · Penicillin · Overexpression · Flux control · *alcA* promoter

Introduction

The first step in penicillin biosynthesis is the production of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) from the three amino acid precursors L-α-aminoadipic acid (Aad), L-cysteine and L-valine by ACV synthetase (ACVS). In the filamentous fungi, Aad is an intermediate in the lysine biosynthetic pathway (Aharonowitz et al. 1992). Isopenicillin N synthase (IPNS) then catalyses the oxidative ring closure of the tripeptide to form isopenicillin N. The final reaction, catalysed by isopenicillin N amidohydrolase/acyl-coenzyme A : 6-aminopenicillanic acid acyltransferase (ACT), removes the L-α-aminoadipate side chain and replaces it with a hydrophobic acyl group such as phenoxyacetyl to form penicillin V, or phenylacetyl to form penicillin G.

The genes for penicillin biosynthesis are clustered together and for *Aspergillus nidulans* and *Penicillium chrysogenum* the entire clusters have been cloned and sequenced (Carr et al. 1986; Ramon et al. 1987; Díez et al. 1990; Montenegro et al. 1990; Smith et al. 1990b, c; MacCabe et al. 1991). The non-penicillin producing fungi *A. niger* and *Neurospora crassa* were found to produce penicillin following transformation with the penicillin gene cluster from *P. chrysogenum*, indicating that the cluster contains all the genetic elements necessary for penicillin biosynthesis (Smith et al. 1990b).

Industrial penicillin production strains of *P. chrysogenum* have been subject to multiple rounds of mutagenesis and screening to produce higher levels of penicillin production. Studies on such strains have revealed the presence of multiple copies of the biosynthetic gene cluster (Smith et al. 1989; Newbert 1994) and higher mRNA levels for all the genes in the cluster (Barredo et al. 1989; Smith et al. 1989, 1990), suggesting that the yield improvements in high titre strains are at least in part the result of the overexpression of the secondary metabolite genes.

Kinetic analyses in the filamentous fungus *Cephalosporium acremonium* C-10 (an industrial production strain) and the actinomycete *Streptomyces clavuligerus* NRRL 3585 (a wild-type strain) have predicted that the production of ACV is a rate limiting step in β-lactam production (Malmburg and Hu 1991, 1992). ACVS has also been postulated to be a rate limiting enzyme by other workers (Zhang and Demain 1991).

Molecular genetic methods have previously been used to enhance β-lactam biosynthetic pathways. An extra copy of the *cefEF* gene, encoding the bifunctional enzyme deacetoxycephalosporin C synthetase/deacetylcephalosporin C synthetase, which catalyses

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the conversion of penicillin N into deacetylcephalosporin C, was introduced into the *cefEF* locus of a cephalosporin production strain of *C. acremonium* by homologous integration (Skatrud et al. 1989; Skatrud 1992). This step was identified as rate limiting since a build-up of penicillin N was observed in this strain. The level of expression of this protein was shown to have increased and, while the overall level of β-lactam products remained unchanged, cephalosporin C production was increased by 15%.

In β-lactam producing strains of actinomycetes, Aad is produced by a different pathway from that in the filamentous fungi. A dedicated pathway, which can be regarded as the first step in penicillin biosynthesis in these organisms, converts L-lysine to Aad. The first enzyme in this pathway is lysine ε-aminotransferase (Madduri et al. 1991). An extra copy of the gene for this enzyme, *lat*, was inserted into the genome of *S. clavuligerus* NRRL 3585 (Malmberg et al. 1993). Recombinant strains containing an extra copy of the *lat* gene were found to produce 2–5 times more β-lactam antibiotic than the wild-type strain. This shows that the production of Aad is rate limiting for β-lactam production in this bacterium.

In *A. nidulans*, the level of Aad, an intermediate of the lysine biosynthetic pathway, is less likely to be rate limiting for penicillin production. In addition to Aad being an intermediate in the lysine biosynthetic pathway it is also released from isopenicillin N upon conversion of the latter to penicillin and this might allow the recycling of Aad back into the penicillin biosynthesis pathway.

The genes for IPNS (*ipnA*) and ACT (*act*) have been overexpressed in *A. nidulans* from the *alcA* promoter (Fernández-Cañón and Peñalva 1995). The levels of transcription from the genes and the respective enzyme activities were found to be considerably higher (40-fold for IPNS and 8-fold for ACT) in the overexpressing strains. The production of penicillin, however, was found to be only slightly increased (ca 25%) when IPNS was overexpressed and was actually decreased when ACT was overexpressed.

The overexpression of the *acvA* gene from *A. nidulans* was desirable for two reasons. The ACVS protein from *A. nidulans* was the first ACVS to be purified (van Liempt et al. 1989), however, the instability of the enzyme in vitro and the low levels produced have hampered further study. Overexpression of ACVS in *Escherichia coli* would be unlikely to yield a completely active enzyme. Previous studies in which peptide synthetase genes and gene fragments have been expressed in *E. coli* have led to the aggregation of the enzyme in inclusion bodies (Krause et al. 1985; Marahiel et al. 1985; Hori et al. 1991; Gocht and Marahiel 1994; Haese et al. 1994; Dieckman et al. 1995). These enzymes have also been found not to keep their full activity, retaining amino acid-dependent ATP-PP_i exchange activity, but losing the ability to produce the peptide products. Peptide synthetases require a cofactor, 4′-phosphopantotheine, which is necessary for the covalent linkage of amino acids to the enzyme and for transpeptidation to occur. Peptide synthetases expressed in *E. coli* have been shown to lack this cofactor and the activity associated with it (Gocht and Marahiel 1994). The use of *A. nidulans* as an expression host for ACVS should overcome these problems. Although the available fungal expression systems cannot at present approach the efficiency of those in *E. coli*, expression in *A. nidulans* is more likely to yield a completely active enzyme with all the correct post-translational modifications. The overexpression of *acvA* also addresses the question of whether ACVS is a rate limiting enzyme for penicillin production, as the production of penicillin in *A. nidulans* can be conveniently monitored using bioassays.

Materials and methods

Strains

The *A. nidulans* strains used were G191 (*fwA1*, *pyrG89*, *pabaA1*, *uaY9*), and JKAβ2 (*yA2*, *metH2*, Δ*acvA*, *argB2*::pJK1 [*argB*$^+$, *alcAp-dom A-lacZ*]. JKAβ2 contains plasmid pJK1 inserted at the *argB2* locus. This plasmid carries a fragment of DNA, *alcAp-dom A-lacZ*, in which a 5′ portion of *acvA* (encoding domain A of ACVS) is fused in-frame to the *lacZ* gene of *E. coli*, under the control of the *A. nidulans alcA* promoter (J. Kennedy et al., in preparation).

The *E. coli* strains used were DH5α, (F$^-$, ϕ80Δ*lacZ* M15, Δ(*lacZYA-argFV169*), *deoR*, *recA1*, *endA1*, *hsdR17* (r_K^-, m_K^+), *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*); BMH 71-18, *mutS*, (*thi-1*, *supE44*, Δ(*lac-proAB*), [*mutS*::Tn*10*], [F′, *proA*$^+$*B*$^+$, *lacI*^{q}*Z*ΔM15]); and JM109, (*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* (r_K^-, m_K^+), *relA1*, *supE44*, λ^-, Δ(*lac-proAB*), [F′, *traD36*, *proAB*, *lacI*^{q}*Z*ΔM15]).

Media

Aspergillus complete medium (ACM) and *Aspergillus* minimal medium (AMM) were used for the growth of *A. nidulans* strains (Pontecorvo et al. 1953). If required, uridine (10 mM, *p*-aminobenzoic acid (1 μg/ml) and methionine (50 μg/ml) were added to the media. Seed culture medium was prepared according to Brakhage et al. (1992) (15 g glucose, 35 g corn steep solids, 5 g $CaCO_3$ made up to 1 l with water and the pH was adjusted to 5.9 with KOH). Fermentation medium (FM) was prepared according to Brakhage et al. (1992) (40 g lactose, 20 g corn steep solids, 10 g $CaCO_3$, 7 g KH_2PO_4, 0.5 g phenoxyacetic acid made up to 1 l with water and the pH was adjusted to 6.0 with KOH). Where glucose was used as a carbon source the lactose was substituted by an equal weight of glucose.

Fermentation conditions

Fermentations were carried out according to Brakhage et al. (1992). Seed and fermentation cultures (20 ml in 250-ml unbaffled flasks), with the addition of the appropriate supplements, were incubated at 26° C and 250 rpm on rotary flatbed shaker. Seed cultures were inoculated with 1 ml of a conidial suspension (10^8/ml) and incubated for 24 h. Mycelia were harvested by centrifugation and washed twice in 0.9% (w/v) sterile NaCl solution and finally resuspended in 14 ml 0.9% (w/v) sterile NaCl solution. FM (20 ml in 250-ml unbaffled

flasks, containing 10 mM cyclopentanone inducer as required) was inoculated with 1 ml of the mycelial suspension. Cultures were incubated for up to 96 h, with flasks being assayed at 24-h intervals. For penicillin bioassays, culture supernatants were collected and for dry weight determinations the mycelia were filtered through Whatman 1MM filter paper, washed with water and dried overnight at 80° C.

Penicillin bioassay

Bioassay agar (150 ml) was melted and cooled to 50° C. *Bacillus calidolactis* C953 slopes were harvested in 2 ml of sterile TES and added to the bioassay agar. This was then mixed and poured into Nunc bioassay dishes (245 mm × 245 mm) on a level surface. Wells were cut into the agar surface using a 9-mm diameter cork borer and culture supernatants (100 µl) and standards (5, 1, 0.5, 0.1, 0.05, 0.01 µg/ml penicillin V) were applied to the wells. This was incubated at 55° C overnight to allow zones of inhibition to form. Penicillin concentrations were determined using a standard curve of concentration against zone diameter on semi-logarithmic graph paper (Brakhage et al. 1992).

Standard DNA techniques

DNA manipulations were carried out as detailed by Sambrook et al. (1989). For small-scale preparation of DNA from *A. nidulans*, the method of Raeder and Broda (1985) was used. Nylon membranes [Biotrans(+), ICN Pharmaceuticals] were used for Southern blot analysis and DNA probes were labelled with α-[^{32}P]dCTP (Redivue, Amersham International) using the Megaprime DNA labelling system (Amersham International). Site-directed mutagenesis was carried out using the Altered Sites in vitro Mutagenesis system (Promega) and DNA sequencing using the Sequenase kit from USB.

Vector construction

The promoter replacement vector, pJKEX, was constructed as shown in Fig. 1a. A 2.65-kb *Bam*HI-*Hin*dIII fragment from pXEP7, containing the start of the *acvA* gene from *A. nidulans*, was subcloned into pALTER-1 (Promega) to produce pJKALT1. In order to clone this into the expression vector pAL3 (Waring et al. 1989), a *Kpn*I restriction site was introduced upstream of the initiation codon by site-directed mutagenesis, using the oligonucleotide shown in Fig. 1b.

When a clone with the correct sequence was identified, using restriction analysis and sequencing from the primer 5′-CATAGCCGCCACTGTAGC-3′, a *Kpn*I-*Bam*HI fragment from this plasmid, pJKALT9, was subcloned into the vector pAL3 to produce pJKEX. The plasmid pAL3 (Waring et al. 1989) contains the *pyr4* gene as a fungal selectable marker and also the *alcA* promoter, without the ATG start codon, allowing genes to be expressed from their own start codon.

β-Galactosidase assays

β-Galactosidase activities were determined as descibed by Brakhage et al. (1992). Activities were measured in crude cell extracts from three separate cultures of each time point.

Protein preparation and protein gel electrophoresis

The method for the preparation for protein extracts for ACVS is adapted from van Liempt et al. (1989) and Zhang and Demain (1992a, b). Mycelia were harvested and washed extensively with water. The mycelia were then frozen with liquid nitrogen and ground to a fine powder with a pestle and mortar. The ground mycelia from a single fermentation flask were then resuspended in 10 ml ACVS buffer A (100 mM MOPS pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 5 mM L-valine, 1 mM EDTA, 50% w/v glycerol) for 30 min with stirring at 4° C. The cell debris was then removed by centrifugation (9000 rpm, 4° C 15 min). Samples (0.1 ml) were made up to 1 ml with 0.5 M TRIS-HCl pH 7.5 and precipitated with 10% trichloroacetic acid (TCA). This was left on ice for 20 min and the protein was harvested by centrifugation in a microcentrifuge (12 000 rpm, 10 min). The pellet was then resuspended in 10–20 µl protein sample buffer and 3 µl 3 M TRIS base. Electrophoretic analysis of proteins was performed in an SDS-polyacrylamide gel (5%) with 3.5% stacking gel according to the method of Laemmli (1970). Proteins were detected by staining with Coomassie Brilliant Blue R.

Glucose assay

The glucose content in media supernatants was determined according to the method of Bergmeyer et al. (1974) using the glucose (HK) reagent from Sigma.

Determination of protein concentration

Protein concentration was determined according to the method of Bradford (1976) using the Biorad protein assay dye reagent, with bovine serum albumin (fraction V, Sigma) as a standard.

Results

Promoter replacement

In order to overcome the difficulties of manipulating a vector containing the whole of the 12-kb *acvA* gene, a promoter replacement strategy was used, in which only the 5′ region of the *acvA* gene was linked to a strong promoter in the plasmid vector pJKEX (Fig. 1). This vector was then used to transform a strain of *A. nidulans* so that integration at the *acvA* locus would result in the replacement of the wild-type promoter with a strong promoter. The *alcA* promoter was used in this study. This well characterised filamentous fungal promoter (Mathieu and Felenbok 1994) allows strong, controllable expression, with the promoter being induced by ethanol, threonine and cyclopentanone and repressed by glucose (Waring et al. 1989; Felenbok 1991).

Transformation and selection of transformants

A. nidulans strain G191 was transformed to uridine prototrophy with plasmid pJKEX. Transformant colonies were purified by streaking out to single colonies twice and genomic DNA was prepared from them. The genomic DNA was then digested with *Hin*dIII and analysed by Southern blot hybridisation using a 2.6-kb *Eco*RI-*Bam*HI fragment from pJKEX as a probe. The

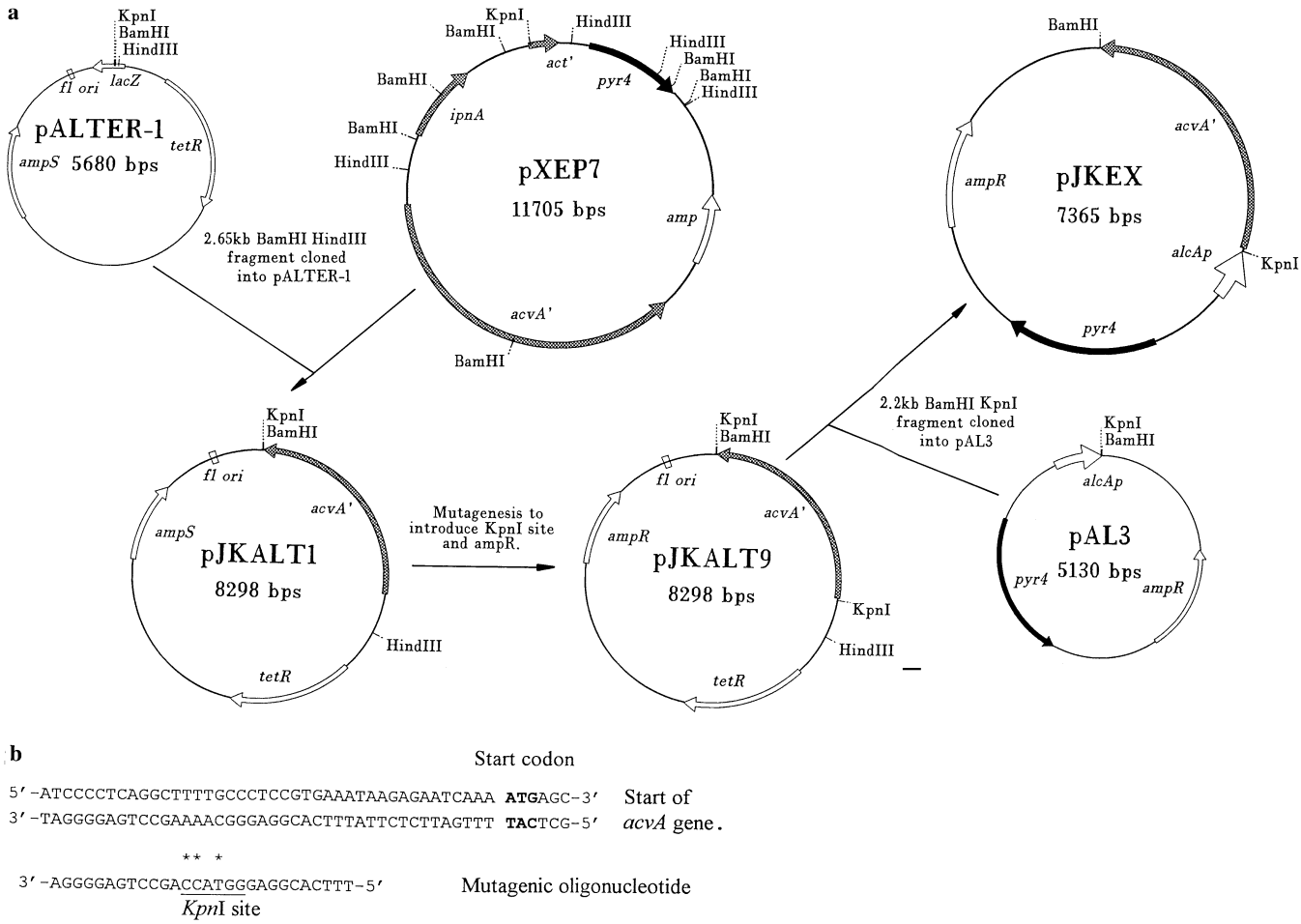

Fig. 1 a Construction of the promoter replacement vector pJKEX. **b** Oligonucleotide for mutagenesis of pJKALT1

integration event and the expected integration pattern is shown in Fig. 2a and the autoradiograph is shown in Fig. 2b.

From this autoradiograph it can be seen that strains JKEX6, 11 and 13 all show the correct pattern of bands for a single copy of pJKEX integrated at the correct locus, while a number of others show patterns consistent with double or multicopy integration events.

Expression of the *alcA* promoter in fermentation conditions

Before fermentations were carried out with the overexpression strains, it was necessary to determine the level of expression of the *alcA* promoter under fermentation conditions. FM medium used for penicillin fermentations is complex, containing corn steep solids and lactose added as a carbon source. Lactose is a non-repressing carbon source for the *alcA* promoter and the corn steep solids contain a large amount of amino acids (Liggett and Koffler 1948) and it was unclear how this would affect the expression. Strain JKAβ2 contains a single copy of a plasmid in which the *alcA* promoter drives a fusion gene consisting of domain A from the *P. chrysogenum acvA* gene and the *E. coli* β-galactosidase gene (Fig. 3a). The construction of this strain will be described elsewhere (J. Kennedy et al., in preparation). Although this construct is integrated into the *A. nidulans* genome at the *argB* locus rather than the *acvA* locus, the expression of the fusion protein will give a good indication of the level of expression expected from the *acvA* overexpression strain under similar conditions. The β-galactosidase activity of the product of this fusion gene can be easily assayed from cell extracts.

Fermentations were carried out with JKDAβ2 in the presence and absence of the inducer (10 mM cyclopentanone). Preliminary experiments showed that cyclopentanone at a concentration of 10 mM fully induced *alcAp*. Flasks were harvested at 0, 24, 48, 72 and 96 h and the mycelia were ground in liquid nitrogen and protein extracts were prepared. The protein extracts were assayed for β-galactosidase activity and protein concentration and the specific activity for β-galactosidase was determined for each flask. Three separate flasks were assayed for each time point.

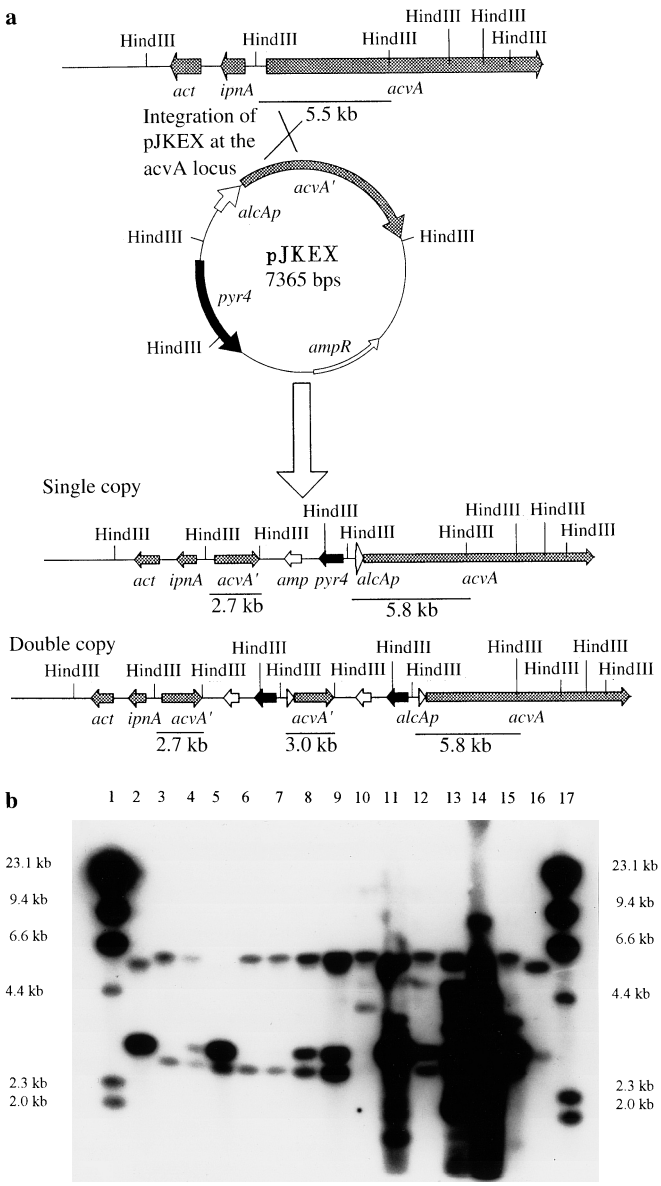

Fig. 2 a Promoter replacement. The band patterns expected following successful promoter replacement, for Southern blots of *Hin*dIII-digested DNA probed with the 2.65-kb *Eco*RI-*Bam*HI fragment containing the start of the *acvA* gene are indicated. **b** DNA from pJKEX transformants (2 μg) was digested with *Hin*dIII, separated in an agarose gel and blotted onto a nylon membrane. The blot was probed with the 2.6 kb-*Eco*RI-*Bam*HI fragment from pJKEX containing the start of the *acvA* gene *Lanes 1* λ *Hin*dIII, *2* JKEX2, *3* JKEX6, *4* JKEX7, *5* JKEX9, *6* JKEX11, *7* JKEX13, *8* JKEX14, *9* JKEX15, *10* JKEX16, *11* JKEX217, *12* JKEX18, *13* JKEX19, *14* JKEX20, *15* JKEX21, *16* G191, *17* λ*Hin*dIII. Strains JKEX6, JKEX11 and JKEX13 show the correct pattern for single copy integration at the correct locus while strains JKEX14, JKEX15 and JKEX18 are double or multicopy integrants. Strain JKEX20, in particular, has a pattern and intensity consistent with the integration of many copies of the plasmid

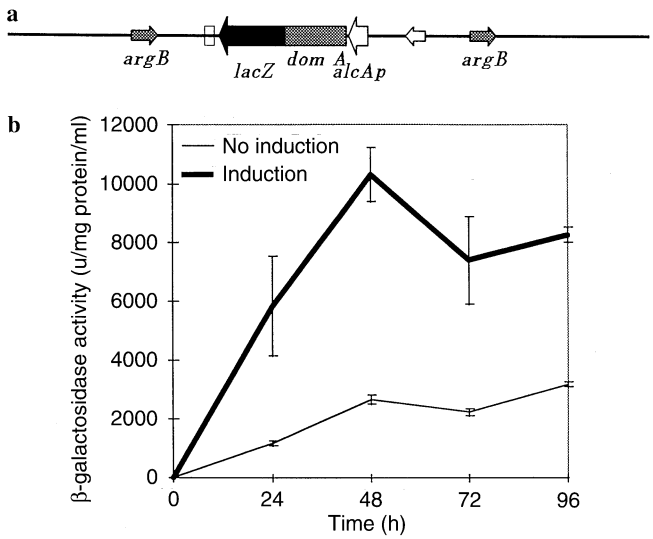

Fig. 3a, b Expression of the β-galactosidase fusion protein. **a** Strain JKAβ2: The *acvA* (domain A) – β-galactosidase fusion protein under the control of the *alcA* promoter (J. Kennedy et al., in preparation). **b** The expression of the *alcA* promoter was analysed in fermentation conditions by assaying strain JKAβ2 for β-galactosidase activity. Fermentations were carried out as described with and without the presence of the inducer cyclopentanone at 10 mM. β-Galactosidase assays were performed on three flasks for each time point and the error bars show the standard error

The results obtained are shown in Fig. 3b. Under non-inducing conditions the maximum expression level of 2500–3000 U/mg protein per ml is reached after 48 h and remains roughly constant thereafter. Under inducing conditions the maximum expression level of 10 000 U/mg protein per ml is reached after 48 h and drops slightly, to approximately 8000 U/mg protein per ml afterwards. The highest level of expression of the *alcA* promoter under fermentation conditions with induction is comparable to the levels of expression found in AMM with induction (J. Kennedy, unpublished results). Induction of the *alcA* promoter under fermentation conditions results in a fourfold increase in the level of expression compared to that of non-induced cultures. It has previously been shown that, when the β-galactosidase gene was placed under the control of the *acvA* promoter at the *argB* locus, the maximum expression level was 100 U/mg protein per ml using identical fermentation conditions (Brakhage et al. 1992). This suggests that the level of ACVS in the cell in the overexpression strain is possibly 25- to 30-fold higher when no induction occurs and 100-fold higher in inducing conditions. Although the different chromosomal locations of the constructs may affect the levels of expression obtained, in the absence of a reliable method for the determination of ACVS activity, this gives an indication of the efficacy of the *alcA* promoter.

Production of penicillin from the *alcAp-acvA* strain

Fermentations were carried out as described with G191 and JKEX6 with and without the addition of cyclopentanone to 10 mM. Flasks were taken for penicillin

bioassays and dry weight determinations at 0, 24, 48, 72 and 96 h with three separate flasks being used for each time point. The results are presented in Fig. 4.

These results clearly show that, under inducing conditions, the overexpressing strain produces around 30 times more penicillin than the wild-type strain. Without induction the overexpressing strain still produces much more penicillin than the wild-type strain (ca eightfold), reflecting the notably elevated expression levels provided by the *alcA* promoter under non-inducing conditions as compared to the *acvA* promoter (see above). The growth rates of the strains showed no significant differences.

Glucose repression of the *alcA* promoter

The *alcA* promoter is strongly repressed by glucose (Felenbok 1991). In order to determine the levels of expression from the *alcA* promoter on glucose, strain JKAβ2 was grown in FM in which the lactose had been substituted by glucose. β-Galactosidase assays were performed on cell extracts at 0, 24, 48, 72 and 96 h, as before, with three separate flasks being assayed for each time point. In addition to this, the media from each flask was assayed for glucose content using the glucose (HK) assay system from Sigma.

The results obtained are shown in Fig. 5a. When glucose is present in the medium at high concentrations, the expression of the fusion protein from the *alcA* promoter is low (<30 U/mg protein per ml). As the glucose is depleted in the later stages of the fermentation, the fusion protein accumulates more rapidly, but

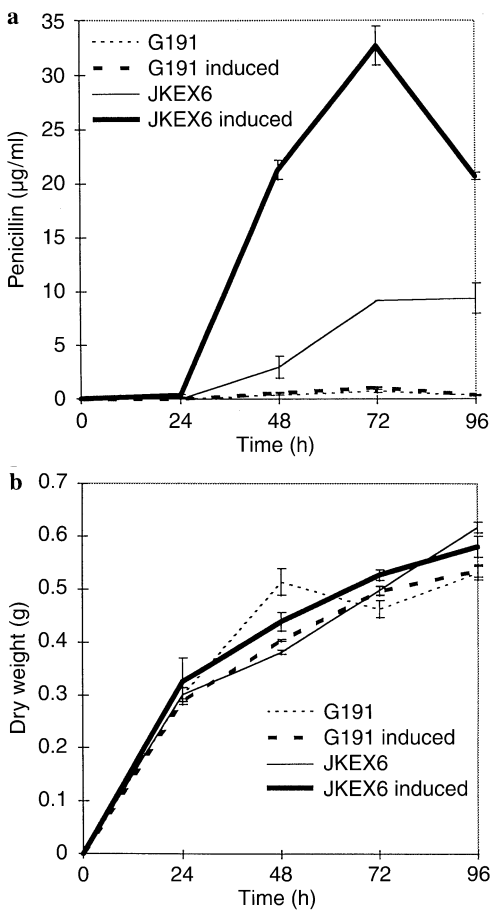

Fig. 4a, b The production of penicillin by strains JKEX6 and G191. **a** Analysis was in fermentation conditions with and without the presence of the inducer cyclopentanone at 10 mM. Penicillin bioassays were performed on the supernatants of three flasks for each time point and the error bars show the standard error. **b** Growth curve for strains JKEX6 and G191 in fermentation media with and without the presence of the inducer cyclopentanone at 10 mM. Dry cell weights were determined for three flasks for each time point and the error bars show the standard error

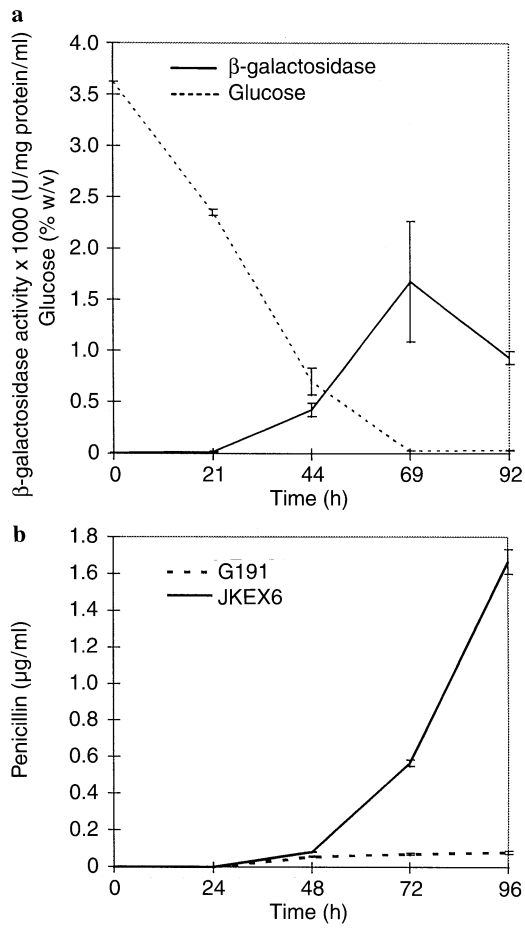

Fig. 5a, b Glucose repression of the *alcA* promoter. **a** The expression of the *alcA* promoter and the utilisation of glucose were determined using strain JKAβ2. This strain was grown in fermentation conditions in which the lactose was substituted by glucose. β-Galactosidase assays were performed on three flasks for each time point and culture supernatants from the same flasks were used for the determination of glucose. The error bars show the standard error. **b** The production of penicillin on glucose was determined for the strains JKEX6 and G191. These strains were grown in fermentation conditions in which the lactose was replaced by glucose. Penicillin bioassays were performed on supernatants from three flasks for each time point. The error bars show the standard error

without reaching the level achieved with cultures grown on lactose.

The penicillin production on glucose of the *acvA* overexpressing strain, JKEX6, and the recipient strain, G191, was then determined. The fermentations were carried out as described, with glucose substituted for the lactose in FM. Penicillin titres were determined from culture supernatants at 24, 48, 72 and 96 h, with three separate flasks for each time point.

The results are shown in Fig. 5. Very little penicillin is produced in both strains in the initial 48 h of fermentation. After this time, the penicillin titre in strain JKEX6 increases while that of G191 remains low (<0.1 μg/ml). This increase in penicillin titre in JKEX6 after 48 h of fermentation corresponds to the depletion of glucose and consequential derepression of the *alcA* promoter, as seen in Fig. 5a.

Detection of the overexpressed ACVS

In order to show that ACVS was being overexpressed from strain JKEX6, total mycelial protein was extracted. The strains JKEX6 and G191 were grown in standard fermentation conditions, with 10 mM cyclopentanone and any necessary supplements, for 48 h, the time point at which the β-galactosidase activity indicated that expression from the *alcA* promoter was at its maximum level (Fig. 3b). Mycelial extracts were prepared and samples analysed by SDS-PAGE (Fig. 6). A strong, high molecular weight band (>205 kDa) can clearly be seen in the extract from strain JKEX6. The apparent size of the protein corresponds well with the original size estimation of *A. nidulans* ACVS of 220 kDa (van Liempt et al. 1989).

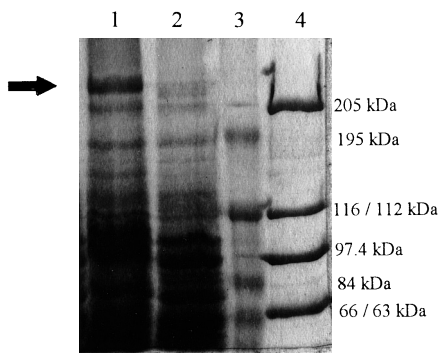

Fig. 6 Detection of δ-(L-α-aminaodipyl)-L-cysteinyl-D-valine (ACV) synthetase in JKEX6. Total mycelial protein was prepared from the strains JKEX6 and G191. An equal amount of protein from each strain was loaded onto a 5% SDS-polyacrylamide gel which was stained with Coomassie blue. The *arrow* marks the position of the ACV synthetase protein. *Lanes 1* JKEX6, *2* G191, *3, 4* molecular weight markers

Discussion

The strategy used for the promoter replacement has been proposed previously (May 1992) though few examples have been reported. This represents a good example of a novel strategy being used for the overexpression of a large gene, which otherwise would have been technically difficult.

Without induction of the *alcA* promoter, the highest penicillin titre observed in JKEX6 is approximately 9 μg/ml. This is about a 12-fold increase over that found in the wild-type strain under the same conditions. Interestingly, the level of penicillin found after 24 h is considerably less in JKEX6 (0.003 μg/ml) than in the recipient strain (0.04 μg/ml). This indicates that the expression of ACVS in JKEX6 is probably less than that from the wild-type promoter in the initial 24 h of fermentation. The penicillin titre, however, increases significantly in the following time points following full derepression of the *alcA* promoter.

In the results from induced JKEX6, the penicillin titre is higher than the wild-type strain at all time points. The highest level was reached after 72 h, when the level of penicillin was found to be approximately 34 μg/ml. This is 30-fold higher than that found in the recipient strain at this time point.

While the terms 'bottleneck' and 'the rate-limiting step' are often used in referring to metabolic pathways, metabolic control theory (Kacser and Burns 1973; reviewed by Kell et al. 1988) proposes that the control of a metabolic pathway is distributed over all the steps of a pathway to varying degrees represented by the flux control coefficient (C_e^j) of an enzyme in the pathway.

If metabolic control theory (Kell et al. 1988) is applied to the penicillin biosynthesis pathway (as shown below), then each enzyme possesses a flux control coefficient, C_e^j.

$$\text{Aad} + \text{Cys} + \text{Val} \xrightarrow[\text{ACVS}]{} \text{ACV} \xrightarrow[\text{IPNS}]{} \text{IPN} \xrightarrow[\text{ACT}]{} \text{Penicillin}$$

C_e^j is defined as the fractional change in flux, ΔJ, divided by the fractional change in enzyme concentration, Δ[e]. If a doubling in enzyme concentration results in a doubling in the flux through the pathway, then this would result in a flux control coefficient of unity for that enzyme. Alternatively, if a doubling of enzyme concentration results in no change of flux, then the flux control coefficient of the enzyme would be zero. In reality, the values for most enzymes lie somewhere between and, for primary metabolic pathways in particular, the flux control coefficients of individual enzymes are usually quite low. Ideally, the measurements for the determination of flux control coefficients should be made in steady-state conditions, in which all parameters, other than the enzyme concentration, are kept

constant. The change in enzyme concentration should also, ideally, be a small increase or decrease.

The flux control summation theorem states that the sum of the flux control coefficients of the enzymes in a metabolic pathway equals unity. For the penicillin biosynthesis pathway this would be expressed by the equation:

$$C^J_{ACVS} + C^J_{IPNS} + C^J_{ACT} = 1$$

The overexpression of IPNS (by 40-fold) in *A. nidulans* resulted in a 25% increase in penicillin production (Fernández-Cañón and Peñalva 1995). The overexpression of ACT (by 8-fold) resulted in a 46% decrease in penicillin production (Fernández-Cañón and Peñalva 1995). These data suggest that C^J_{ACT} and C^J_{IPNS} are close to zero. If the flux control summation theorem is applied in this situation, then it would suggest that C^J_{ACVS} approaches unity.

There is no reliable quantitative method for the direct measurement of the level of ACVS. However, the relative levels of β-galactosidase in strain JKAβ2 in induced and non-induced conditions (Fig. 3b) give an indication of the increase in expression from the *alcA* promoter in these conditions. At the 72-h time point, there is a 3.3-fold increase in β-galactosidase activity in the induced cultures compared to the non-induced cultures. If the penicillin titres at 72 h from JKEX6 in induced and non-induced conditions are compared (Fig. 4a) then there is a 3.5-fold increase in penicillin production. These results would imply that C^J_{ACVS} is close to unity. This result fits in very well with the data obtained for IPNS and ACT.

The exact determination of flux control coefficients for the enzymes requires a more sophisticated approach involving measurement of the effects on flux of small changes in enzyme concentration. Such an approach would be possible with the *alcA* promoter since its expression can be controlled by repression with glucose and induction with cyclopentanone. However, a reliable, quantitative assay for ACVS would also be necessary.

The results obtained with glucose demonstrate that the *alcA* promoter is under tight control, with penicillin production being almost completely abolished until the glucose concentration falls to derepressing levels. The results obtained with the strain G191 on glucose differ significantly from results obtained under similar conditions with strain AXB4A (Brakhage et al. 1992). Glucose was found to repress penicillin biosynthesis in the latter study, but only by about 50%, whereas with strain G191, the repression is about 90%. This represents an unexpected strain difference, with penicillin production in G191 being much more sensitive to glucose repression. Strain G191 carries a mutation in the *uaY* gene. This gene encodes a transcriptional activator for a number of unlinked genes involved in purine utilisation (Suarez et al. 1995). It is possible that this transcriptional activator is somehow involved in the regulation of penicillin production.

The overproduction of penicillin in the overexpressing strain demonstrates that the ACVS in this strain retains its full activity. This shows that the cofactor, 4′-phosphopantotheine, is correctly added to the overexpressed enzyme. The expression, in *A. nidulans*, of peptide synthetase enzymes and enzyme fragments should therefore yield proteins which retain their full potential activity, overcoming a problem encountered with *E. coli* expression systems.

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