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On the production of α,β-heterodimeric acyl-coenzyme A: isopenicillin *N*-acyltransferase of *Penicillium chrysogenum*

Studies using a recombinant source

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A high level *E coli* expression system has been constructed for the *Penicillium chrysogenum penDE* gene, which encodes the acyl-coenzyme A: isopenicillin *N*-acyltransferase (AT) enzyme. Induction of overexpression of recombinant AT (recAT) by increasing the growth temperature of the host adversely affected solubility and activity of the AT enzyme. Addition of isopropylthio-β-D-galactopyranoside (IPTG) at decreased growth temperatures (less than 32°C) resulted in the overproduction of soluble, active recAT. When purified to homogeneity, recAT was an α,β-heterodimer, comprised of 11 kDa (α) and 29 kDa (β) subunits, derived from a 40 kDa precursor polypeptide by a posttranslational cleavage. The recAT enzyme contained both the acyl-coenzyme A: isopenicillin *N*-acyltransferase and the acyl-coenzyme A: 6-aminopenicillanic acid acyltransferase activities. The processing event that generated the two subunits of recAT from the 40 kDa precursor polypeptide occurred between Gly^{102}/Cys^{103}. This expression system produced a large amount of soluble, active recAT that is identical to native AT, making it a suitable source of AT enzyme for further characterization.

Acyltransferase; Penicillin biosynthesis; Isopenicillin N; Benzylpenicillin; *Penicillium*; *Aspergillus*; *E. coli*

1. INTRODUCTION

The final step in the biosynthesis of β-lactam antibiotics in *Penicillium chrysogenum* and *Aspergillus nidulans* involves the removal of the L-α-aminoadipoyl side chain of isopenicillin N (IPN) and exchange with one of many non-polar side chains (such as phenylacetyl, resulting in benzylpenicillin) [1–4]. This reaction might proceed directly, or as a two step process, in which 6-aminopenicillanic acid (6-APA) is formed as an intermediate [3]. The enzyme catalyzing this reaction, acyl-coenzyme A: isopenicillin N acyltransferase (AT), was purified and found to be an α,β-heterodimer containing subunits of 11 and 29 kDa [5]. In both *P. chrysogenum* and *A. nidulans*, the gene encoding AT, *pen*DE, is located immediately adjacent to the *pcb*AB (δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine synthetase (ACVS)) and *pcb*C (IPN synthase) genes [6,7]. Cloning and sequencing of the *P. chrysogenum* [8–10] and *A. nidulans pen*DE gene [10,11] revealed that they are colinear, and are ~73% identical at the amino acid level. The *P. chrysogenum pen*DE gene is composed of four exons and is transcribed into a ~1.4 kb mRNA. The message is apparently translated into a 40 kDa precursor polypeptide, which is posttranslationally cleaved, generating subunits of 11 kDa (α subunit) and 29 kDa (β subunit).

The difficulties involved in purifying reasonable quantities of AT from native sources has resulted in a diversity of opinions concerning the active form of the enzyme, kinetics, and substrate profile [4,5,8–10]. A reliable heterologous expression system for AT would greatly facilitate meaningful characterization and analysis by providing large amounts of AT enzyme. Early attempts at overexpression of AT in *E. coli* relied on a plasmid in which the *pen*DE gene was driven by the λP_L promoter, resulting in mostly insoluble recAT production [10,12].

In this paper, we report the design and construction of an heterologous *E. coli* expression system which results in the soluble overproduction of active recAT. Physical analysis indicates that this recAT is identical to AT purified from *P. chrysogenum*, making the recombinant source an ideal system for obtaining large amounts of AT.

2. EXPERIMENTAL

2.1. *Microorganisms, media, and growth conditions*

E. coli strains TG1 (*supE,hsdΔ5,thi,Δ(lac-proAB)*,F'[*traD*36, *proAB*$^+$,*lacI*,*lacZΔ*M15]), and JM109(*recA*1,*supE*44,*endA*1, *hsdR*17, *gyrA*96,*relA*1,*thi,Δ*(*lac-proAB*),F'[*traD*36, *proAB*$^+$,*lacI*^{q},*lacZΔ*M15]) [13] were grown in 2 × TY broth [14] supplemented with ampicillin (80 μg · ml^{-1}), kanamycin (50 μg · ml^{-1}), or chloramphenicol (30 μg · ml^{-1}) under standard conditions [14], except as noted. *Micrococcus luteus*

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NCTC 8340 and *Staphylococcus aureus* NCTC 6571 were grown according to standard microbiological techniques.

2.2. *Bioassay of enzyme activities*

2.2.1. Phenylacetyl-coenzyme A: 6-APA acyltransferase

The protein solution for assay contained 50 mM Tris-HCl, pH 7.8, 5 mM dithiothreitol (DTT), 1 mM phenylacetyl-CoA and 0.2 mM 6-APA. After incubation at 30°C for 15 min, the reaction was stopped by the addition of an equal volume of cold methanol and assayed for benzylpenicillin by the hole-plate assay method [15] using *M. luteus* NCTC 8340.

2.2.2. Phenylacetyl-coenzyme A: IPN acyltransferase

The assay mixture was identical to that described above, except that 6-APA was replaced by 1 mM IPN. After 1 h, the reactions were diluted 1:8 with methanol (50% final concentration of methanol). Production of benzylpenicillin was assessed by the hole-plate assay method [15] using *S. aureus* NCTC 6571.

2.3. *Molecular biological and other techniques*

DNA techniques followed standard procedures [13]. Plasmid DNA maxipreparation was performed using Qiagen anion exchange columns as recommended by the manufacturer (Qiagen, Inc., Studio City, CA, USA). Oligonucleotide linker sequences were synthesized using an Applied Biosystems DNA Synthesizer (model 380B or 394). Bacterial transformation was accomplished either by the calcium chloride method [13], or by electroporation, using a Bio-Rad Gene Pulser as recommended by the manufacturer (Bio-Rad Laboratories, Richmond, CA, USA). Computer analysis of DNA and protein sequences was performed using Macvector software (IBI, New Haven, CT, USA).

2.4. *Plasmid constructions*

The *pen*DE gene used was derived from plasmid pOW1068 [10]. Removal of 3′ noncoding sequences was achieved by digestion of plasmid pOW1068 with *Nsi*I and *Bam*HI. The ~4.1 kb *Nsi*I/*Bam*HI fragment containing the vector and the *pen*DE gene was isolated, and then ligated with an *Nsi*I/*Bam*HI linker, as illustrated in Fig. 1, creating plasmid pMAT3. The *pen*DE gene was removed from pMAT3 on a ~1.1 kb *Nde*I/*Bam*HI fragment and ligated into the ~4.5 kb *Nde*I/*Bam*HI fragment from plasmid pRH1090 [16], creating the *E. coli* expression plasmid pMAT4 (Fig. 1).

2.5. *Expression of recAT from* E. coli *JM109/pMAT4*

E. coli JM109/pMAT4 was grown in shake flasks to mid-log phase at 25°C, 250 rpm. The culture was diluted 1:50 with fresh medium and induced for protein production by the addition of IPTG (0.01–1.0 mM), or by increasing the incubation temperature (25–37°C), or both. Crude cell extracts were prepared from cell pellets resuspended in glycerol, followed by the addition of 50 mM Tris-HCl, pH 7.8, 5 mM DTT. The suspension was then sonicated, and in the case of cleared cell lysates, spun at 20,000 × *g* for 30 min at 4°C. Protein was analyzed by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [17], and by bioassay, as described above.

2.6. *Fermentation and purification of recAT from* E. coli *JM109/pMAT4*

E. coli JM109/pMAT4 was grown in a New Brunswick Scientific (MPP-40) 30 l fermentor at 30°C, in 2 × TY supplemented with 0.1 mM IPTG and 30 $\mu g \cdot ml^{-1}$ chloramphenicol, using standard techniques. Harvested cells were frozen, resuspended as described above, and disrupted by sonication. The crude lysate was cleared by centrifugation, nucleic acids were removed by polyethylenimine (PEI) precipitation, and the supernatant was subjected to HPLC/FPLC chromatographic purification on Q-Sepharose Fast Flow (Pharmacia), Gel Filtration (Superdex 200 Prep. Grade; Pharmacia), and Rainin Hydropore AX columns. Activity was followed by monitoring for phenylacetyl-CoA: 6-APA acyltransferase activity, and by SDS-PAGE analysis. Full details of the purification procedure will be reported elsewhere [18].

2.7. *NH_2-terminal amino acid sequencing*

NH_2-terminal amino acid sequences were obtained from purified recAT, after separation of the subunits by SDS-PAGE, followed by electroblotting onto Immobilon PVDF membrane (Millipore UK). Both protein bands were excised and sequenced directly.

2.8. *Electrospray mass spectrometry (ESMS)*

Electrospray mass spectrometry was performed on purified recAT and recorded on a VG BIO-Q instrument. The mass scale was calibrated with myoglobin.

3. RESULTS

3.1. *Analysis of overexpression of recAT in* E. coli *JM109/pMAT4*

The effect of IPTG concentration (0–1.0 mM IPTG) was examined in shake flask experiments over the course of 18 h post-induction growths, at 25°C, 30°C, and 37°C. In the absence of IPTG, recAT production was constitutively low at 25°C and 30°C, whereas at 37°C, a large amount of insoluble 40 kDa polypeptide was produced. At 0.01–1.0 mM IPTG concentrations, a high level expression of soluble 29 kDa and 11 kDa subunits was observed at 25°C and 30°C. At 37°C, only the high level expression of insoluble 40 kDa polypeptide is observable by SDS-PAGE, similar to that seen in the absence of IPTG. In the absence of plasmid, *E. coli* JM109 does not produce the 11 kDa, 29 kDa, or the 40 kDa species, verifying that they derive from pMAT4.

The expression of soluble 11 kDa and 29 kDa subunits correlates with the presence of both the acyl-CoA: 6-APA AT and the acyl-CoA: IPN AT activity. The cells obtained from 37°C cultures contained a small amount of these activities, however the activities peak early in the growth (~3 h) and decline in cultures induced over a longer period, despite the continued accumulation of the 40 kDa polypeptide (Fig. 2A).

Overexpression of soluble, active recAT was observed over an 18 h time course (post-induction) in the presence of 0.01–1 mM IPTG at both 25°C and 30°C. Fig. 2B illustrates (by SDS-PAGE analysis) the soluble accumulation of 11 kDa and 29 kDa subunits over time in 30°C growths with 0.01 mM IPTG. The occurrence of these subunits correlates directly with the presence of AT activity detected in bioassays.

3.2. *Purification and analysis of recAT*

Purification of recAT was performed by following AT activity and analysis of protein fractions by SDS-PAGE. The resultant purified recAT consists of two subunits of 11 kDa and 29 kDa (Fig. 3). Both subunits are consistently present in purified recAT, and both acyl-CoA: 6-APA AT and acyl-CoA: IPN AT activities are present, mirroring the situation of AT purified from native sources [5].

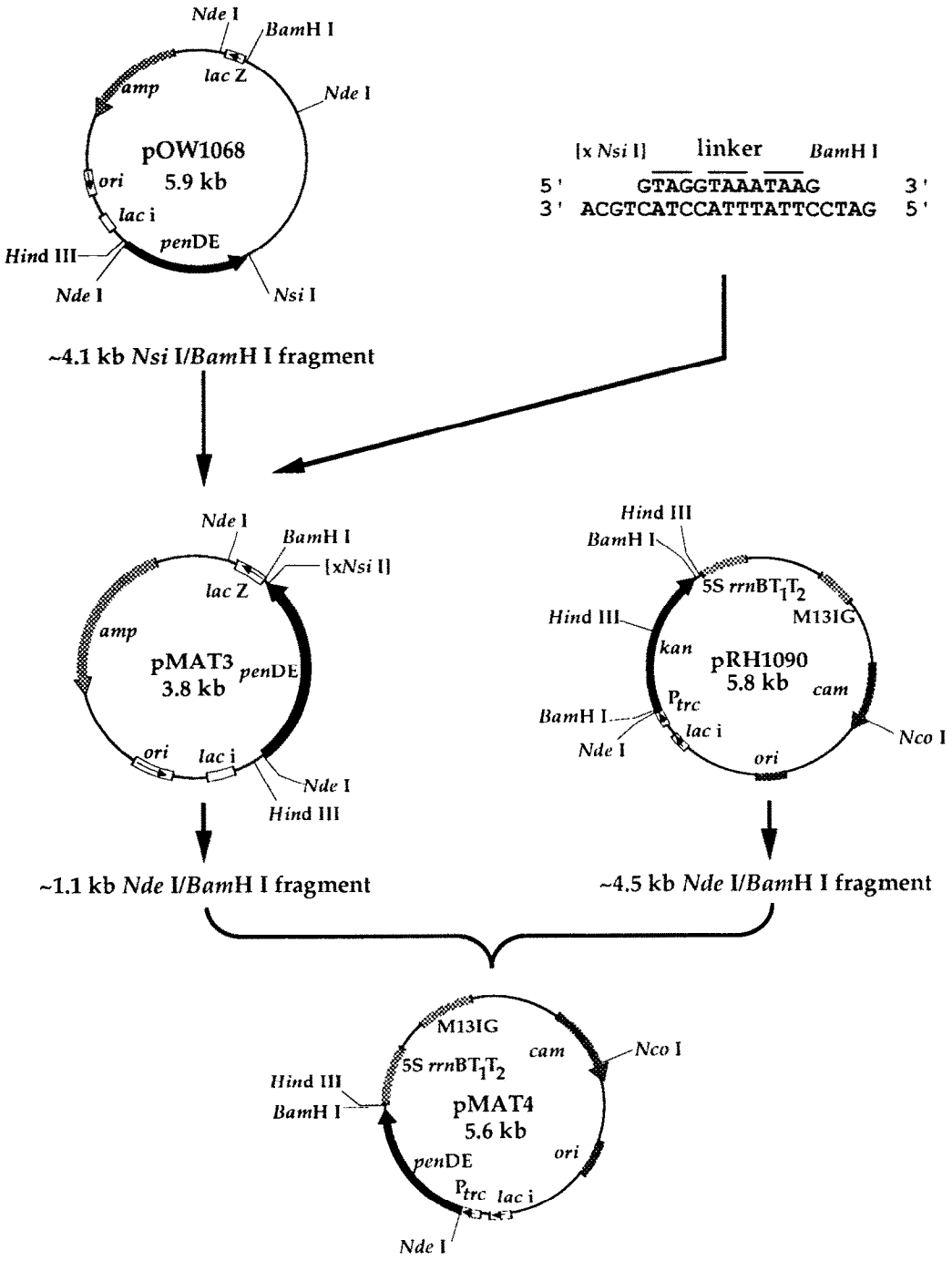

Fig. 1. Construction of *E. coli* expression plasmid pMAT4.

3.3. *Analysis of recAT by NH_2-terminal amino acid sequencing and electrospray mass spectrometry*

The presence of the two subunit, α,β-heterodimer in purified recAT indicates that posttranslational cleavage of the 40 kDa preprotein has occurred during its production in *E. coli*. Either the recAT contains this activity, or the host cell contains a protease that cleaves recAT. It was therefore of primary importance to establish the position of the cleavage site in recAT. Each subunit was subjected to NH_2-terminal amino acid sequencing, and these sequences were compared to those from native AT [5]. In both cases, the sequences were identical: NH_2-Met-Leu-His-Ile-Leu-Cys-Gln-Gly-Thr-Pro for the 11 kDa α subunit, and NH_2-(Trp)-Thr-Thr-Ala-Tyr-Cys-Gln-Leu-Pro-Asn for the 29 kDa β subunit. Interestingly, both the recAT and native AT show

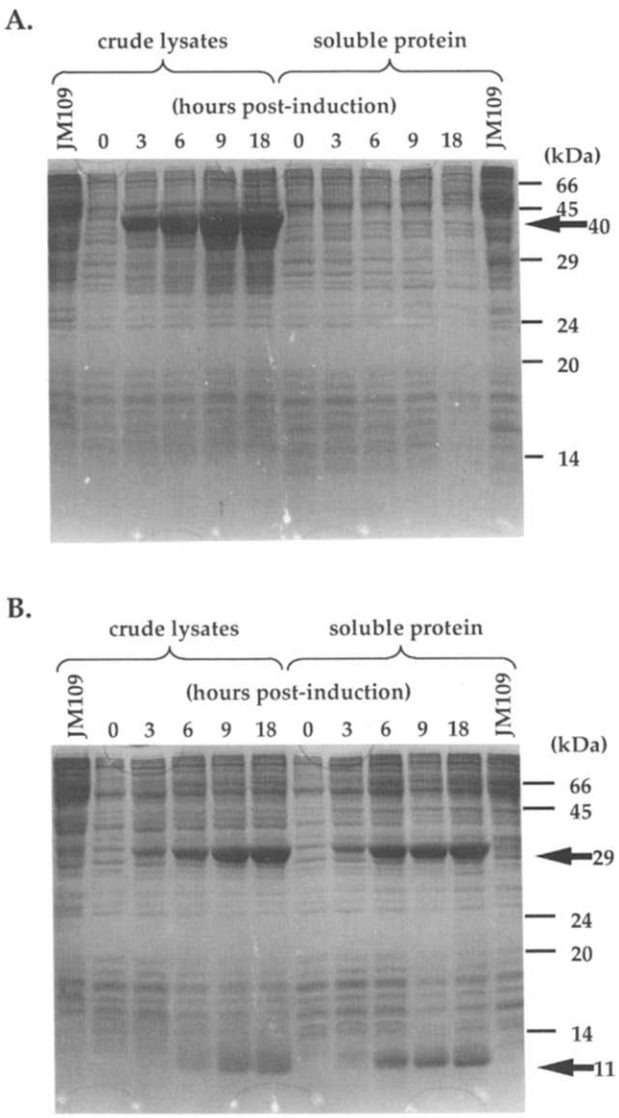

Fig. 2. 15% SDS-PAGE analysis of recAT expression from JM109/pMAT4, from 0 h to 18 h post-induction. Lanes labelled JM109 contain no plasmid. (A) Induction at 37°C, no IPTG. (B) Growth at 30°C, induced for protein production with 0.01 mM IPTG.

the first residue as Trp, whereas Cys is encoded by the DNA sequence. (The *A. nidulans* 29 kDa subunit sequences correctly as Cys [5]).

Evidence against the presence of a Trp residue at the NH_2-terminus of the β subunit was gained from an analysis of the same purified recAT fraction by electrospray mass spectrometry. The mass obtained for each subunit using this technique (11,498.24 ± 1.07 Da, α subunit; 28,462.18 ± 4.68 Da, β subunit) closely agrees with the calculated mass based on DNA sequence (11,498 Da, α subunit; 28,457 Da, β subunit). ESMS also revealed the presence of two minor species having masses of 16 and 32 Da greater than the major β subunit species. It is likely that these species result from some form of cysteine or methionine sulphur oxidation (data

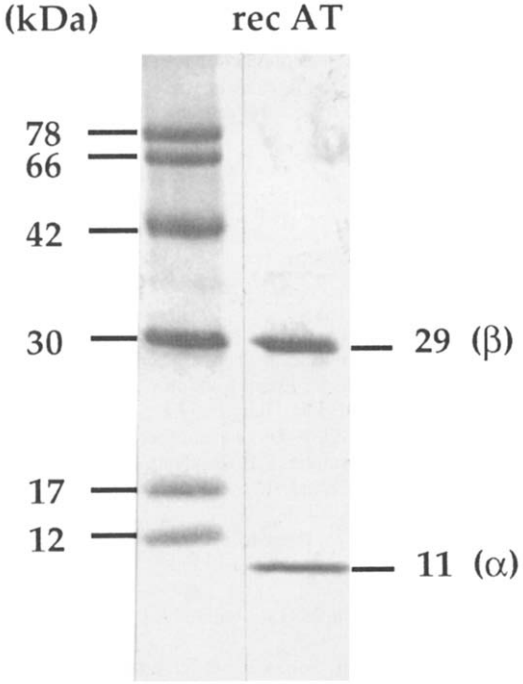

Fig. 3. Purified active recAT (15% SDS-PAGE).

not shown). The mass of the major β subunit species as determined by ESMS, however, is consistent with Cys, not Trp, being the first residue. These observations indicate that a specific cleavage of the recAT 40 kDa preprotein occurs between Gly^{102} and Cys^{103}, and further indicate that recAT is identical to AT purified from *P. chrysogenum*.

4. DISCUSSION

The soluble overexpression of recAT in *E. coli* using plasmid pMAT4 results in the production of an α,β-heterodimer, containing subunits of 11 kDa and 29 kDa. Purified recAT has both acyl-CoA: 6-APA AT and acyl-CoA: IPN AT activities. A posttranslational event creates this configuration by cleaving a 40 kDa precursor polypeptide between Gly^{102}/Cys^{103}, as observed in AT enzyme isolated from *P. chrysogenum* [5]. Production and purification of recAT from fermentations should generate a sufficient amount of recAT enzyme for complete kinetic analysis, substrate specificity profiling and crystallographic studies.

An investigation into the mechanism of cleavage of the 40 kDa polypeptide will also be undertaken. It is possible that the inactive 40 kDa precursor polypeptide, or the active 11 kDa + 29 kDa recAT (or either subunit) contains the specific cleavage activity that generates the mature form of the recAT enzyme. If it is encoded by recAT (or the preprotein), we will attempt to determine whether the cleavage is an intramolecular or intermolecular event.

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