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# HIGH-LEVEL SOLUBLE EXPRESSION AND PURIFICATION OF DEACETOXYCEPHALOSPORIN C/ DEACETYLCEPHALOSPORIN C SYNTHASE.

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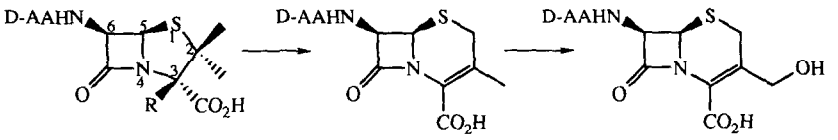
*ABSTRACT.*

The deacetoxycephalosporin C/deacetylcephalosporin C synthase gene from *Cephalosporium acremonium* has been expressed at high levels in *E. coli* under control of the *trc* promoter in a vector modified by PCR. The enzyme is produced in soluble, highly active form in contrast to previously reported expression in insoluble form under control of the $\lambda P_L$ promoter. A three step purification of this unstable enzyme is reported.

*INTRODUCTION.*

A key step in the biosynthesis of cephalosporins is the ring-expansion of penicillin N (1a) to deacetoxycephalosporin C (2) (Fig. 1). This reaction and the subsequent hydroxylation to deacetylcephalosporin C (3) are both catalysed by the enzyme deacetoxycephalosporin C/ deacetylcephalosporin C synthase (DAOC/DACS) in the eukaryote *Cephalosporium acremonium*.




(1a) R=H
(1b) R=D

(2)

(3)

D-AA = δ-(D-α-Aminoadipoyl)

**Figure 1.**

In the prokaryote *Streptomyces lipmanii* the two steps of ring expansion and hydroxylation are catalysed by separate enzyme activities. The genes for all three of these enzymes have been cloned and expressed in insoluble form under the control of the $\lambda P_L$ promoter in *E. coli* by scientists at Eli Lilly and Co..[1,2,3] The substrate specificity of *C. acremonium* DAOC/DACS from this recombinant source has been shown to be the same as

the wild-type enzyme[4]. In earlier work we observed that a related enzyme, isopenicillin N synthase (IPNS) was produced insolubly from the $\lambda P_L$ promoter but solubly from the *trc* promoter on high copy-number pUC-derived vectors[5,6]. In this paper we describe the construction of a similar *trc* promoter vector for the DAOC/DACS gene and the purification of the soluble gene product.

*VECTOR CONSTRUCTION.*

The DAOC/DACS gene was conveniently available as a 1.2 kb *Nde* I, *Bam* HI fragment from pIT511[1], the ATG start codon of the gene being part of the *Nde* I restriction site (5'-CA$^{v}$TATG-3'). Our previous IPNS expression vectors were constructed to express genes on *Nco* I, *Pst* I or *Hin* dIII fragments and thus mutagenesis was required to express the DAOC/DACS gene most expeditiously. The correct spacing between the start codon and the ribosome binding site and promoter is important for high level expression. Of the various ways that could be used to effect the desired changes, we decided to employ the polymerase chain reaction (PCR). Our strategy for vector construction is shown in Fig. 2.

Plasmid pJB500[5] was used as template for normal PCR using primers RJH 1 and RJH 2[7]. This PCR amplifies the *trc* promoter region and changes the *Nco* I site to a *Nde* I site; in addition the *Pst* I site is replaced by sites for *Bam* HI and *Sma* I whilst the *Hin* dIII site is retained. The amplified 311bp band was purified by agarose gel electrophoresis, restricted with *Eco* RI and *Hin* dIII and subcloned into M13mp8 for sequencing[8]. An M13 clone of verified sequence was restricted with *Eco* RI and *Hin* dIII and the 300bp fragment was reinserted into the parent plasmid pJB500, giving pRH103[9]. To facilitate further manipulation, a kanamycin resistance genblock (Pharmacia) was subcloned into the unique *Bam* HI site of pRH103 giving pRH107. This insertion allows double digests with *Nde* I and *Bam* HI to be monitored easily and provides a useful selectable marker for subsequent subcloning. The *trc* promoter/kanamycin resistance gene/transcriptional terminator unit was then subcloned as a 1.7kb *Ssp* I fragment into pJS63[10] to give pRH1090. At 37$^{0}$C pRH1090 was found to direct synthesis of very high levels (>50% of soluble cell protein, data not shown) of kanamycin phosphotransferase in *E. coli*[11]. Finally, ligation of the DAOC/DACS gene to the large fragment of *Nde* I, *Bam* HI digested pRH1090 gave pRH1091.

*EXPRESSION.*

In previous reports we have shown that *E. coli* strains JM109 and NM554 are suitable hosts for expression of recombinant proteins off the *trc* promoter[5,6] and we evaluated these two strains with pRH1091 for production of soluble DAOC/DACS. At 37$^{0}$C in NM554 the plasmid pRH0191 underwent a deletion of 116 bp (detected by double stranded DNA sequencing) in the region of the promoter and no expression was observed. This deletion is similar in nature to a deletion we observed during the course of our work on IPNS expression systems[6] although in that case expression was still high after the deletion. In JM109, however, no deletion was observed and at 37$^{0}$C the DAOC/DACS gene was expressed to the extent of about 5% of soluble cell protein with insoluble material accumulating with time. At 30$^{0}$C, with

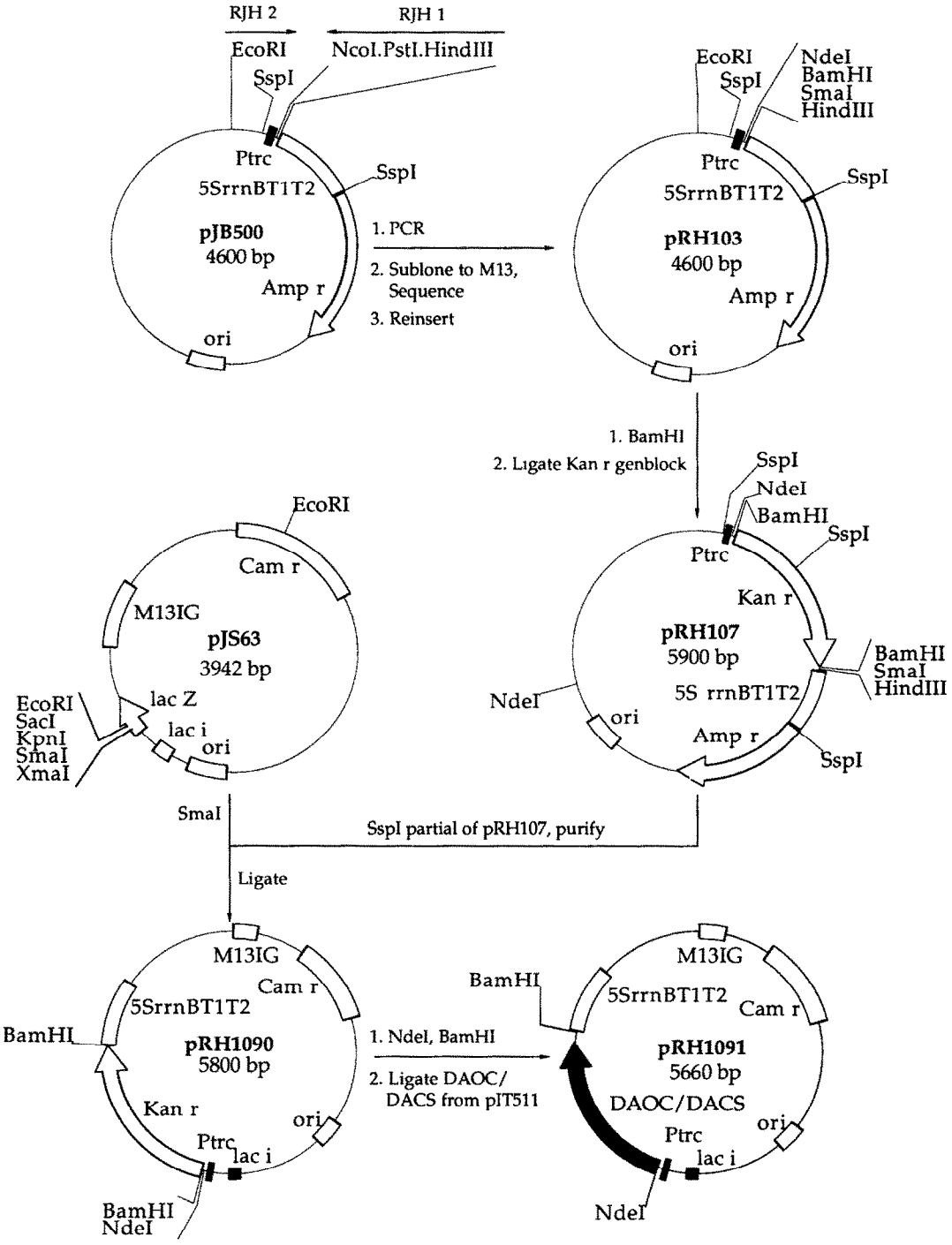



**Fig. 2 Construction of DAOC/DACS expression vector**

IPTG induction, the expression was optimal at about 20% of soluble cell protein (Fig. 3) with minimal insoluble protein. Activity of crude cell lysates[12], as adjudged by bioassay[13], was extremely high ($20x10^{-3}$ I.U.mg$^{-1}$ as compared to *ca.*$10x10^{-3}$ I.U.mg$^{-1}$ for material purified to apparent homogeneity[14] from the previously reported insoluble recombinant source[1]). Fermentation has proved reproducible on a 30 litre scale and we can routinely obtain 20g of crude protein with a specific activity of 20 to $40x10^{-3}$ I.U.mg$^{-1}$ from such fermentations.

*PURIFICATION.*

Both wild type and previously expressed recombinant DAOC/DACS have been reported to be unstable and difficult to purify[1,13,15]. The crude material produced in this study is also unstable and our purification procedures have, thus far, been aimed at producing highly purified material rather than optimising recovery. The loss of activity of crude and partially purified enzyme correlates with the appearance of a *ca.* 30kDa degradation product and several smaller (<5kDa) peptides. N-terminal sequencing of one of these smaller peptides reveals it to be a seven residue internal peptide flanked by tryptic-like cleavage sites. We have not yet unequivocally demonstrated that this cleavage is catalysed by an *E. coli* protease, since there exists the possibility that it is an intrinsic property of DAOC/DACS (further studies will be reported elsewhere). These instability problems notwithstanding, a standard 3 column FPLC purification procedure produces multi-milligram quantities of highly purified enzyme which appears to be stable when flash-frozen and stored at -80$^0$C, Fig.3.

| Purification Step | Total Protein (mg) | Total Activity x$10^{-3}$ I.U. | Yield (%) | Specific Activity x$10^{-3}$ I.U.mg$^{-1}$ | Purification (-Fold) |
|---|---|---|---|---|---|
| Crude Extract | 3,100 | 62,000 | 100 | 20 | - |
| Q-Sepharose | 150 | 11,250 | 18 | 75 | 4 |
| S-200 | 77 | 7,700 | 12 | 100 | 5 |
| Mono-Q | 43 | 5,100 | 8 | 150 | 7.5 |

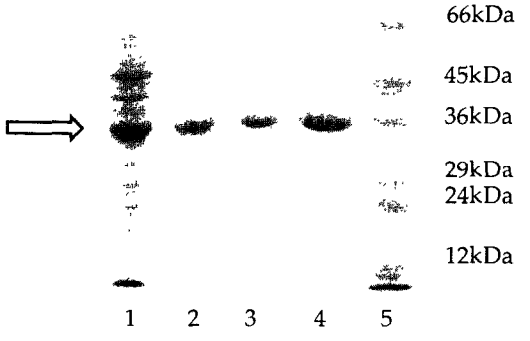



**Fig. 3 Purification of DAOC/DACS.**
The results of the purification are shown in the table above and gel profile to the left.
Lane 1: Crude Extract.
Lane 2: Q-Sepharose.
Lane 3: S-200.
Lane 4: Mono-Q.
Lane 5: Molecular weight markers.
The position of DAOC/DACS is shown by the arrow.

*CONCLUSIONS.*

The results presented in this paper provide another example of the recently observed effect of different promoters on the solubility of recombinant gene products[5,6]. In addition, the occasional deletion of one of two tandem, homologous promoters which we observed with an IPNS expression vector is now shown to be a more general phenomenon in such dual promoter systems. The production of soluble, highly purified DAOC/DACS paves the way for biophysical and mechanistic studies of this key enzyme. Preliminary investigations of the substrate specificity (for example with the deuterated substrate, (1b)) of DAOC/DACS from this new source are reported in the following paper.

*ACKNOWLEDGEMENTS.*

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7. Oligonucleotide primers were synthesised by Val Cooper using standard phosphoramidite chemistry on an Applied Biosystems 380B DNA Synthesiser and had the following sequences:
   RJH 1: 5'-CCCAAGCTTCCCGGGATCCAGCCCCATATGGTCTGTTTCCTGTGTGAAAT-3',
   RJH 2: 5'-GGCCAGTGAATTCTCATGT-3'.
8. Single stranded M13 DNA was sequenced by the dideoxy-method using Sequenase 2.0$^{TM}$ and universal primer, 8 out of 12 clones had the expected sequence.
9. All plasmids were characterised by fine structure restriction mapping.
10. pJS63 is a derivative of the pUC119-derived pJS62[5] produced by restricting with *Bam* HI and *Hin* dIII and filling-in with the Klenow fragment of DNA pol I. This removes the polylinker cloning sites from *Bam* HI to *Hin* dIII inclusive. The fidelity of this operation was confirmed by DNA sequencing.

11. Cells transformed with pRH1090 and pRH1091 are grown at 27$^0$C when expression is not required, since the plasmid copy-numbers and expression levels are lower.
12. Soluble protein was released from cells by treatment with lysozyme/DNase I on a small scale, and by sonication on a large scale. Lysates were cleared by centrifugation before bioassay. One international unit (I.U.) is defined as the amount of protein required to convert penicillin N to one nmole of cephalosporin C per minute under the reaction conditions.
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