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HIGH-LEVEL SOLUBLE EXPRESSION OF ISOPENICILLIN N SYNTHASE ISOZYMES IN *E. COLI*.

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ABSTRACT.
The Isopenicillin N Synthase genes from *Cephalosporium acremonium, Penicillium chrysogenum* and *Aspergillus nidulans* have been expressed at high levels in *Escherichia coli* under control of the *trc* or *lac* promoters. The enzymes are produced in soluble form in contrast to previously reported expression in insoluble form under control of the λP_L promoter.

INTRODUCTION.

A key step in the biosynthesis of penicillins and cephalosporins is the four-electron oxidative cyclisation of the tripeptide, L-α-aminoadipoyl-L-cysteinyl-D-valine (ACV) to isopenicillin N[1]. This remarkable transformation is catalysed, in β-lactam antibiotic producing fungi and bacteria, by the enzyme Isopenicillin N Synthase (IPNS). Studies in these laboratories[1] have revealed a relaxed substrate specificity for the enzyme from *Cephalosporium acremonium* and over 40 tripeptides have been shown to be converted into a plethora of product structures. Certain substrates, for example the tripeptides in which valine has been replaced by either allylglycine or by α-aminobutyrate, give more than one product, upon incubation with the enzyme and cofactors (Fig.1).

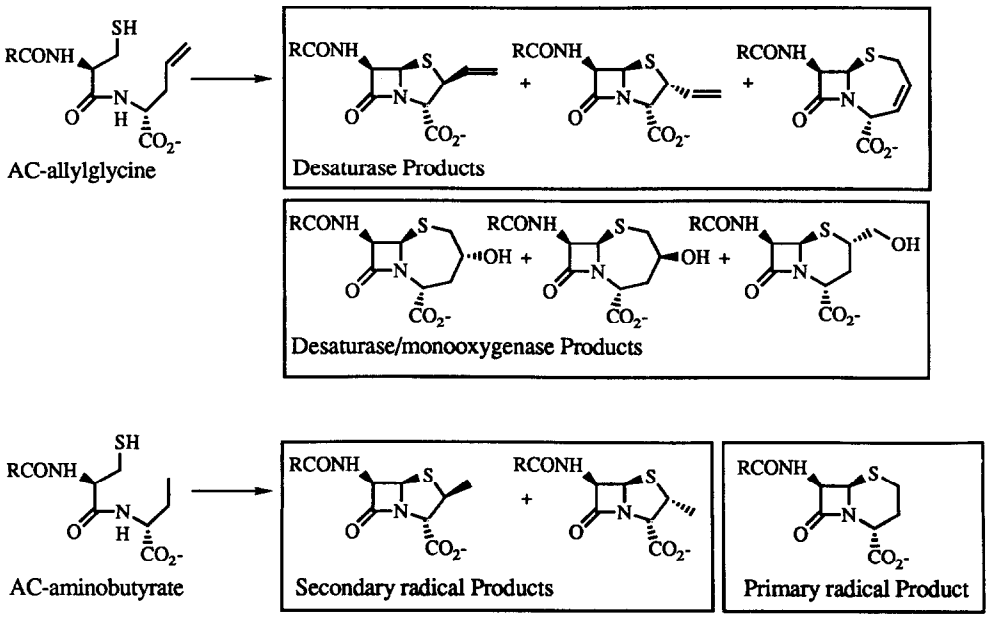

FIG. 1. Processing of unnatural substrates by cIPNS.

We have been interested in ascertaining whether the different products from a particular substrate are indicative of active-site mediation of chemistry or are simply a manifestation of fundamental chemical reactivity principles. Thus it would appear reasonable to suggest that the ratio of penam to cepham products with the α-aminobutyrate tripeptide is a consequence of the relative stabilities of primary *versus* secondary radicals[2]. In the case of the allylglycine containing tripeptide the products arise from two different chemistries, desaturation and mixed function desaturation/monooxygenation[3], and it could be postulated that this is due to an active-site effect, enzyme geometry influencing the ratio of the two chemistries.

Several approaches to investigate this possible dichotomy can be envisaged. If the product composition of a reaction is relatively unaffected by various active-site alterations then presumably the product composition is a consequence of fundamental chemical reactivity principles. In contrast, if product composition is differentially affected by active-site alteration the implication is that the active-site make-up affects the reaction to an extent beyond the mere promotion of the high-energy chemistry that IPNS carries out[4]. In the absence of a structural model for IPNS and with only limited data as to which residues constitute the active-site[5] it is difficult to predict which site-directed mutations might alter the active-site in the aforementioned manner without destroying enzyme activity altogether[6]. Active IPNS has been isolated from many sources and the isozymes have been shown to have significantly different amino acid sequences[7,8,9,10,11,12,13]. It is likely that some of the regions of strong homology are essential for catalytic activity and are presumably thus in the active-site (or distant but crucial from a structural maintenance point of view). Other residues in the active-site are of lesser importance and change might be tolerated as regards processing of the natural substrate. The isozymes have evolved to process the natural substrate and it is unlikely that evolutionary pressure has been brought to bear on the processing of unnatural substrates. Thus the isozymes comprise a class of IPNS variants which are all active towards the natural substrate but which have not been preselected on the basis of their ability to handle unnatural substrates (this is important to ensure that any comparison is not intrinsically predisposed to give one result or the other). The minor active-site perturbations assumed from the sequence heterology of the isozymes are therefore exactly the sorts of mutations which we require to test our assumptions concerning the chemistry of this enzyme.

We have chosen, for initial comparison, the enzymes from the fungi *C. acremonium* (cIPNS), *Penicillium chrysogenum* (pIPNS) and *Aspergillus nidulans* (aIPNS) and from the bacterium *Streptomyces lipmanii* (sIPNS). The genes encoding these enzymes have been cloned and sequenced by several groups and we and others have reported expression studies using various promoters(Table 1).

TABLE 1
Previous expression systems for IPNS isozymes

Source	Pro	Level	Form	Activity	Ref.
C. acremonium	*trp*	15-20%	soluble	✓	7
C. acremonium	λP_L	~20%	insoluble	✓ (low)	14
C. acremonium	*trc*	low	soluble (?)	×	15
C. acremonium	*trc*	~5%	soluble	✓	16
P. chrysogenum	*trp*	~10%	soluble	✓	8
P. chrysogenum	λP_L	low	insoluble (?)	?	9
A. nidulans	λP_L	10-15%	insoluble	✓ (low)	10
A. nidulans	*lpp*	major protein	soluble (?)	✓	11
S. lipmanii	λP_L	10-15%	insoluble	✓ (low)	10

For our purposes, gram quantities of easily purifiable, soluble, active enzymes are required and previously described expression systems are inadequate. Work from these laboratories has shown that the enzyme from *C. acremonium* can be suitably expressed in *Escherichia coli* under the control of the *trc* promoter[16]. We report here further studies with the *C. acremonium* enzyme and production of expression systems for the enzymes from *P. chrysogenum* and *A. nidulans*. The possible reasons for differences in solubility of the same enzymes expressed from different promoters are discussed.

EXPRESSION OF THE C. ACREMONIUM IPNS GENE.

Initial studies with this enzyme involved the expression vector pIT337 in which the cIPNS gene is under control of the *trp* promoter in a temperature-regulatable copy number vector[7]. cIPNS was produced to the extent of 15-20% of total soluble protein depending on the strain of *E. coli* used[17]. Subsequent problems with the *trp* promoter necessitated the use of an alternate expression system and workers at Eli Lilly constructed pIT353 in which the gene is under control of the λP_L promoter in a plasmid containing the gene, cI857, for a temperature sensitive repressor. *E. coli* transformed with pIT353 and induced at 42°C produces cIPNS to the extent of *ca.* 20% of total cell protein but in this case the enzyme is in the form of insoluble inclusion bodies[14] (only low levels of activity can be recovered from these inclusion bodies). We have expressed cIPNS from the *trc* promoter in a series of pKK233-2-derived vectors and observed soluble expression, the level of which correlates with plasmid copy-number[16]. On the basis of this we subcloned the *trc*-promoted cIPNS gene as a 1.8kb *Bam* HI/*Eco* RI fragment into the multiple cloning sites of the high copy-number plasmid pJS62[16] (pUC119[18] in which the β-lactamase gene has been inactivated by insertion of a chloramphenicol resistance gene at the unique *Sca* I site) and pJB507 (pUC119 in which the β-lactamase gene has been inactivated by insertion of a kanamycin resistance gene[19] into the unique *Sca* I site) giving pJB506 and pJB508 respectively (construction of all new cIPNS expression vectors is outlined in Fig.2). The reason for producing two similar vectors differing in their antibiotic resistance markers was that we intended exploiting the ability of these phagemids to produce single-stranded DNA for sequencing and mutagenesis. The commonly used helper phage M13KO7[18] is selected for in the presence of kanamycin and the *E. coli* strain, CJ236, used for mutagenesis by the procedure of Kunkel *et al.*[20] has a episomal chloramphenicol resistance gene. Uracil-containing single stranded DNA for mutagenesis using the procedure of Kunkel *et al.* can be produced using pJB508, CJ236 and the helper phage R408[21] while single-stranded DNA for other mutagenesis procedures can be produced using pJB506 and M13KO7 in any appropriate strain. In the event both systems proved useful, M13KO7 proving a superior helper phage. Using single-stranded phagemid DNA over 2.5kb of the 5.7kb vector pJB506, including all junctions created in the construction, was sequenced. A small number of errors were encountered in the previously published sequences of the chloramphenicol resistance gene[22], the M13 intergenic region[23] and the downstream region of the cIPNS gene[7]. These errors are listed in Table 2.

The pUC origin of replication in pJB506 and pJB508 carries a temperature-sensitive mutation and copy number increases approximately 10-fold upon shifting temperature from 27 to 37°C. In both NM554[24] and JM109[25] cIPNS expression from pJB506 and pJB508 was temperature inducible. At 27°C no cIPNS was apparent by SDS-PAGE or by bioassay whereas at 37°C very high-level, *soluble* expression was observed (Plate 1). We estimate the level of expression to be of the order of 25% of total soluble cell protein on the basis of SDS-PAGE and enzyme activity measurements both with crude and purified material[16]. The yield of cIPNS from NM554/pJB506 was approximately 100 mgl^{-1} and other strain/plasmid combinations gave similar high-yields.

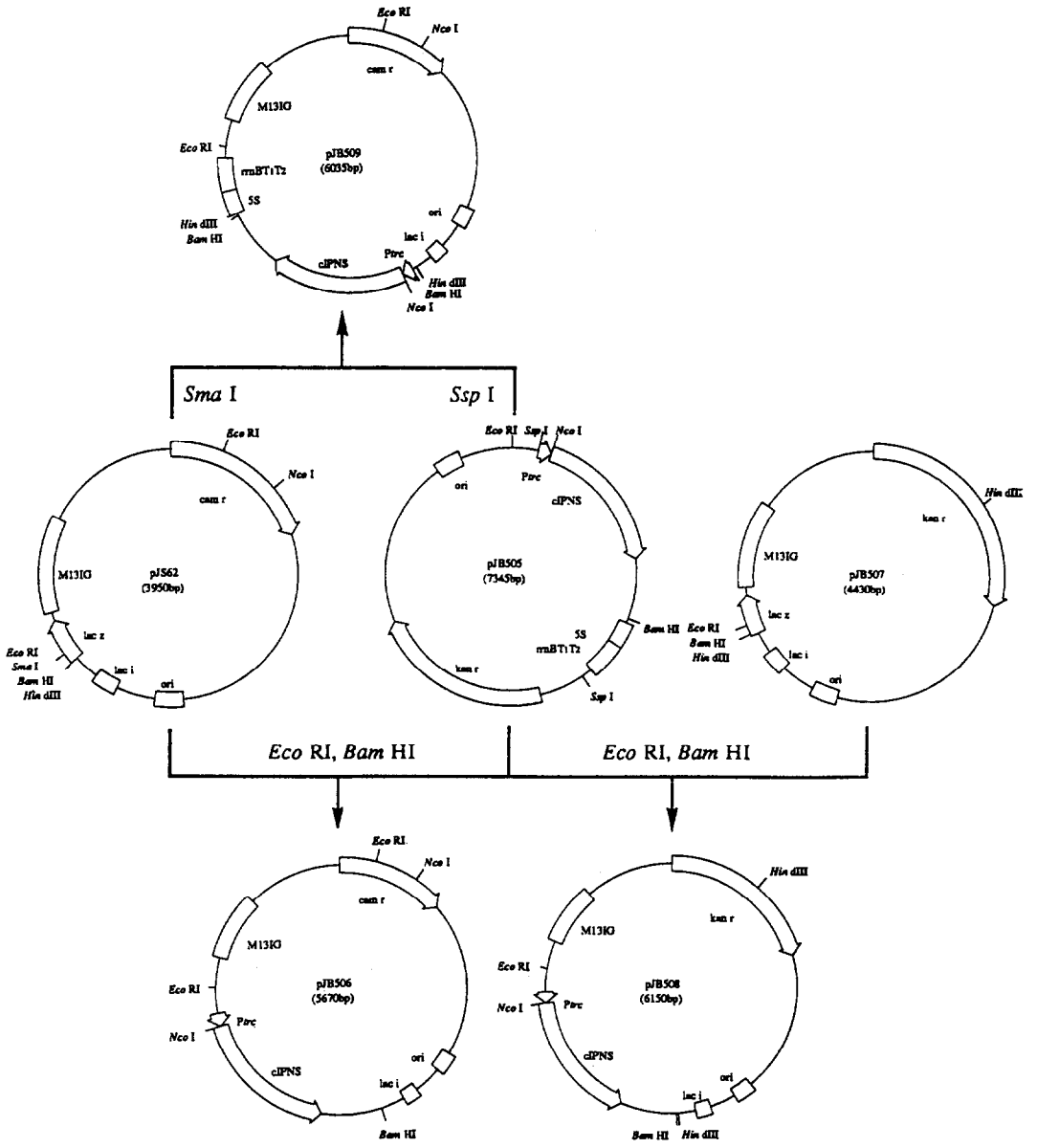

FIG. 2. Construction of cIPNS expression vectors.

TABLE 2

Sequence Errors Encountered

Region	Ref.	Errors	Effect
Chloramphenicol Resistance Gene	22	C622-T substitution T insertion at 737	Silent Non-coding
M13 Intergenic Region	23	C5820-T substitution C5869-T substitution CG deletion at 5491-5492	Domain B of (+) strand origin of replication Silent in pUC, alters C-terminus of Gene IV fragment in M13
cIPNS Fragment	7	A insertion at 1551 G1538-A substitution G1496-C substitution Published 3'-terminus GGATCCGGGGA ATC should read GGATCGGGGATCC	Downstream sequence, may affect transcription.

The activity in crude extracts was up to 1 I.U./mg total protein and mass spectral analysis[16] revealed the cIPNS to be correctly N-terminally processed. In order to investigate whether transcriptional terminators proximal to the 3'-terminus of the gene had any effect on expression, an additional plasmid was constructed. Thus, the *trc*-promoted cIPNS gene along with transcriptional terminators was excised from pJB505[16] as a 2kb *Ssp* I fragment and subcloned into the *Sma* I site of the multiple cloning-site of pJS62. The resultant plasmid, pJB509, had the cIPNS in the opposite orientation to pJB506 and pJB508. cIPNS expression from NM554/pJB509 was very similar to the expression from systems lacking the transcriptional terminators and the opposite transcriptional direction appears to have little effect.

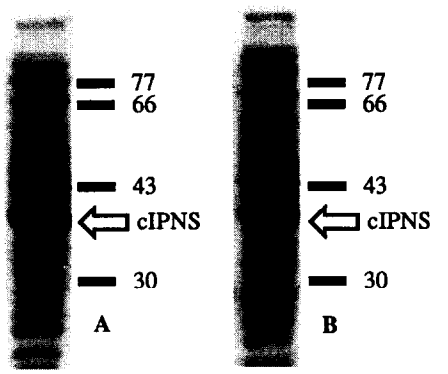

PLATE 1. Expression of cIPNS.
Lysates of expression stains were analysed by SDS-PAGE followed by staining with Coomassie Blue. Molecular weight markers are in kD (see text) (A) NM554/pJB506, (B) NM554/pJB508.

EXPRESSION OF THE P. CHRYSOGENUM IPNS GENE.

The pIPNS gene was obtained from a derivative (pPSJ75) of pXL2[8] as a 1kb *Nco* I fragment and was subcloned into the unique *Nco* I site of pJB501[16] giving pJB601, in which the gene is in the correct orientation with respect to the *trc* promoter (construction of pIPNS expression vectors is outlined in Fig.3). The *trc*-promoted pIPNS gene along with transcriptional terminators was isolated from pJB601 as a 1.6kb *Ssp* I fragment and subcloned into the *Sma* I site of the multiple cloning site of pJS62. The resultant plasmid, pJB603, had the pIPNS gene in the same orientation as the cIPNS gene in pJB509. NM554/pJB603 expressed pIPNS in soluble form at the relatively low level of 2-3% of total soluble cell protein (Plate 2). The yield of pIPNS was approximately 10-15 mgl^{-1} and the activity of crude extracts, using an assay procedure optimised for cIPNS, was of the order of 0.02 I.U./mg total protein. To investigate the reasons for the relatively low levels of expression of

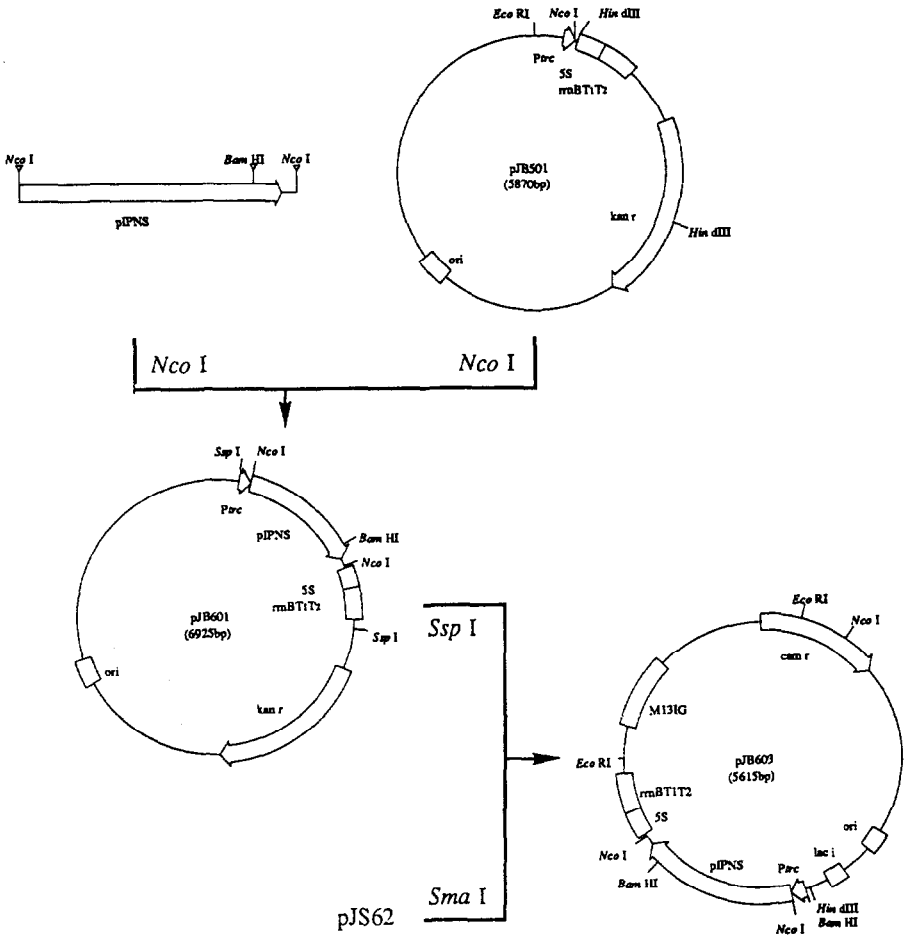

FIG. 3. Construction of pIPNS expression vectors.

pIPNS we first confirmed the plasmid construction by double-stranded DNA sequencing. A single run revealed that a 250 base pair stretch from 5' to the *trc* promoter into the pIPNS gene was exactly as predicted. Extensive restriction mapping showed that the 3'- end of the gene was intact. Northern analysis (data not shown) probing with PCR amplified cIPNS and pIPNS gene fragments indicated that messenger levels were similar for NM554/pJB506 and NM554/pJB603 expression systems. Thus we conclude that the low expression is not due to an incorrect construction or to low levels of transcription. Barredo *et. al.*[9] have identified a short inverted repeat in the early region of the pIPNS gene. These authors speculated that this hairpin-loop might interfere with translation; the results reported here support this hypothesis. This mRNA secondary structural problem could presumably be rectified by mutation at the DNA level. We have not performed this mutational analysis as the system NM554/pJB603 satisfies our criteria for expression (*vide supra*).

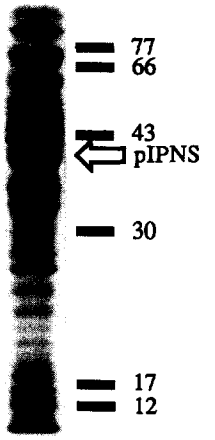

PLATE 2. Expression of pIPNS from NM554/pJB603

EXPRESSION OF THE A. NIDULANS IPNS GENE

The aIPNS gene was isolated as a 1.9kb *Nco* I fragment from pOW216[10] and subcloned into the unique *Nco* I site of pJB501. The plasmid with the aIPNS gene in the desired orientation was designated pJB700 (construction of all aIPNS expression vectors is outlined in Fig.4). The *trc*-promoted aIPNS gene along with the transcriptional terminators was subcloned as a 2.5kb *Ssp* I fragment into the *Sma* I site of pJS62. Transformation of JM109 gave two plasmids differing in the orientation of the insert. The plasmid with the *trc* promoter acting in synergy with the *lac* promoter from pJS62 was designated pJB701, the other plasmid, pJB702.

Transformation of our usual expression strain NM554 with pJB701 followed by growth at 37°C repeatedly gave 10^4-fold fewer transformants than expected. Following a growth of one of these transformants to assess for aIPNS expression (*vide infra*) we checked the integrity of the plasmid and noticed that one of the three anticipated *Bam* HI sites was not present. Fine structure restriction mapping revealed a deletion of *ca.* 150bp in the region of the plasmid multiple cloning site, 5' to the insert. Plasmid isolated from NM554/pJB701 transformed and grown at 27°C did not show signs of deletion and experiments in which cultures of NM554/pJB701 were shifted from 27°C to 37°C showed that this deletion is temperature inducible (data not shown). The nature of the deletion in the plasmid designated pJB703 was then further characterised by double-stranded DNA sequencing. This revealed that the deletion from pJB701 was 143bp in length with end points over a 42bp window (Fig.5). The deletion end points cannot be given with certainty because of the sequence identity between the 3'-ends of the *lac* and *trc* promoters.The net result is a splicing of the tandem lac^{18} and trc^{26} promoters from pJB701 to give a perfectly reconstructed *lac* promoter in pJB703. The mechanism whereby this deletion takes place in the *rec* A$^-$ strain NM554 is not known and attempts to induce the mutation in JM109 proved unsuccessful.

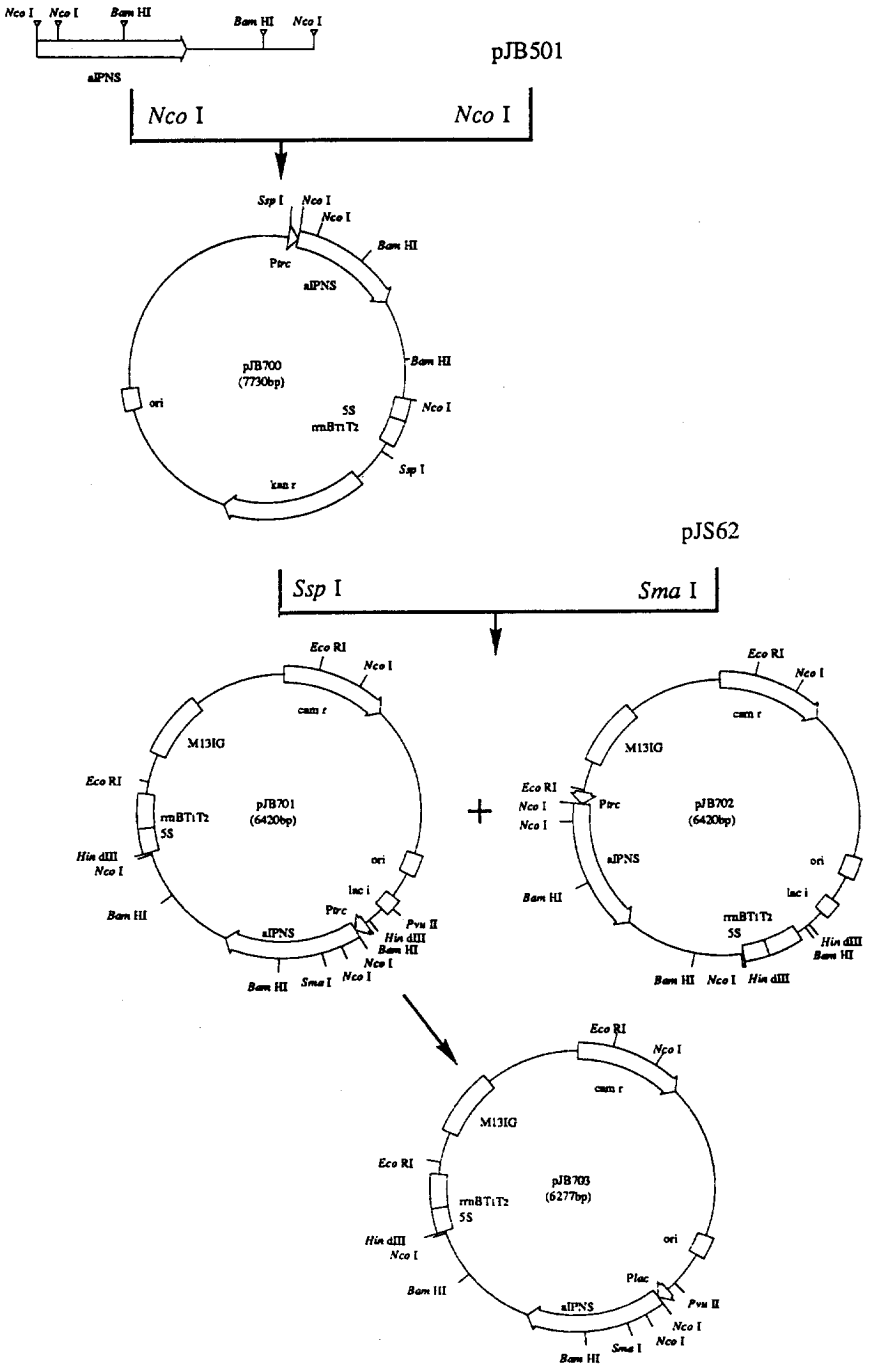

FIG. 4. Construction of aIPNS expression vectors.

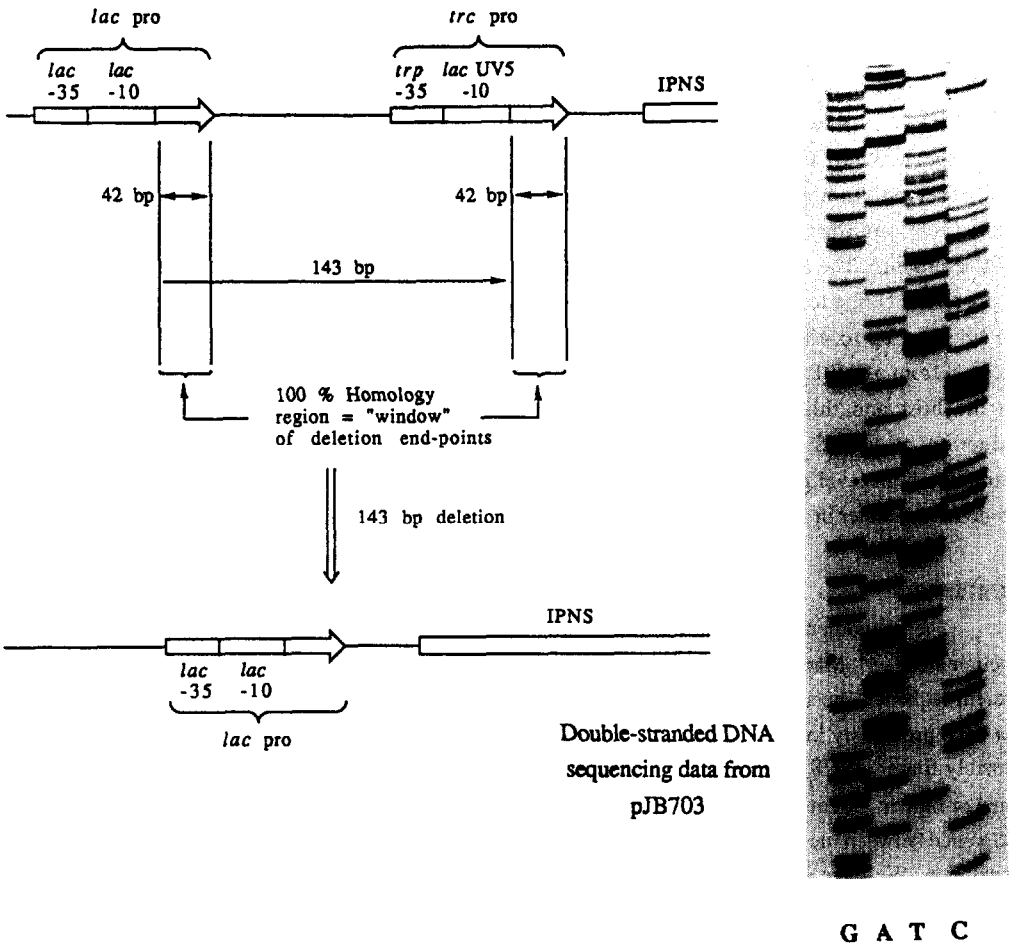

FIG. 5. Deletion of a promoter from pJB701 giving pJB703.

Sequence of the tandem promoter region of pJB701 (Predicted and confirmed by double-stranded DNA sequencing); the *lac* promoter is shown in **bold**, the *trc* promoter in *italics* and the start codon of a IPNS gene in ***bold italics***, the 42bp 100% homology regions of deletion end-points are underlined , the deletion removes 143 bp reconstituting a perfect *lac* promoter (see above):

ATT AAT GTG AGT TAG CTC ACT CAT TAG GCA CCC CAG GCT TTA CAC TTT ATG CTT CCG
GCT CGT ATG TTG TGT GGA ATT GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC
TAT GAC CAT GAT TAC GCC AAG CTT GCA TGC CTG CAG GTC GAC TCT AGA GGA TCC CCA TTC
TGA *AAT GAG CTG TTG ACA ATT AAT CAT CCG GCT CGT ATA ATG TGT GGA ATT GTG* AGC GGA
TAA CAA TTT CAC ACA GGA AAC AGA CC ***ATG*** GGT TCA GTC AGC
Met Gly Ser Val Ser

We then assessed the levels of aIPNS expression from these vectors (Plate 3). Levels were high (approximately 10-15% of soluble protein) with JM109 transformed with pJB701, pJB702, or pJB703 and grown at 37°C. With NM554 similar expression was observed from pJB702 but greatly enhanced expression was observed from pJB703 (>40% of soluble protein). The activity of crude extracts was as high as 1.5 I.U./mg total soluble protein and yields of aIPNS were up to 400 mgl^{-1}. We presume that the deletion from pJB701 confers some growth advantage on the host and this is most likely achieved by reduction of aIPNS expression. Nonetheless the extremely high level of expression of aIPNS from a single *lac* promoter in NM554/pJB703 is noteworthy.

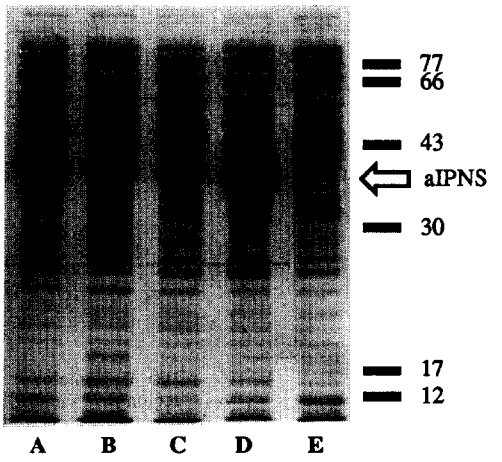

PLATE 3. Expression of aIPNS.
(A) JM109/pJB701, (B)JM109/pJB702, (C) JM109/pJB703, (D) NM554/pJB703, (E) NM554/pJS62

SUMMARY

The phenomenon of inclusion body formation with over-expressed foreign proteins in *E. coli* is well documented[27,28]. Many possible reasons for insolubility have been discussed *inter alia* growth, temperature, protein sequence, metal ion binding, and high production rate. The effect of different promoters on solubility has not been previously investigated to the best of our knowledge. We have observed in this work that three (and possibly four[29]) IPNS isozymes that are expressed in *insoluble* form off the λP_L promoter are expressed to the same or higher extent in *soluble* form off the *trc* or *lac* promoters. This is a bizarre observation given that the difference between the two promoters would be expected to be at the level of transcription, and not at the level of translation. We have examined the composition of inclusion bodies from cells expressing IPNS from a λP_L system and found them to contain, in addition to IPNS, RNA, plasmid DNA, and what we presume to be ribosomal proteins (data not shown). It is possible that translation takes place on nascently transcribed RNA and that aggregation of such polysome/transcription complexes leads to precipitation and formation of inclusion bodies. Why then does the same situation not prevail with an alternate, but equally "strong", promoter? We are thus far unable to offer an experimentally verified answer to this question but suggest that transcriptional speed (being a product of promoter binding and polymerase efficacy) is the crux. The *trc* promoter perhaps directs synthesis of RNA at a rate such that the polysome/transcription complex does not precipitate. Experiments to investigate this hypothesis are in progress. In addition the differential solubility phenomenon is being investigated for other proteins. It remains for us to produce a soluble expression system for sIPNS before we can carry out the substrate handling properties alluded to in the introduction.

MATERIALS AND METHODS

Strains and growth conditions

E. coli strains NM554 [*recA*1, *araD*139, Δ(*araABC-leu*) 7697, Δ*lacX*74, *galU*$^-$, *galK*$^-$, *hsdR*, *hsdM*$^+$, *rspL*, *strA*, *thi*, *mcrA*(-), *mcrB*(-)], JM109 [*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*), {F', *traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15}] and TG1[30] [*supE*, *hsd*Δ5, *thi*, Δ(*lac-proAB*), {F', *traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15}] were used throughout this work. Transformed strains were grown in 2xTY (1.6%

tryptone, 1.0% yeast extract, 0.5% NaCl) supplemented with $Fe(NH_4)_2SO_4$ (150μgml^{-1}). Ampicillin (50 μgml^{-1}), chloramphenicol (20μgml^{-1}) or kanamycin (50μgml^{-1}) were added as appropriate. Small scale cultures were grown in shake flasks at 27°C or 37°C for *ca.* 15 h.

DNA manipulation and plasmids

All DNA manipulations were carried out essentially according to Maniatis *et al.*[30] pUC119 was the generous gift of J. Vieira. Kanamycin resistance 'genblock' (Tn903) and M13KO7 helper phage were obtained from Pharmacia. pOGO216 and pPSJ75 were provided by Eli Lilly and Co. Restriction enzymes and DNA modifying enzymes were from Pharmacia, NBL, New England Biolabs and Cambridge Bioscience. Radioactive nucleotides were from Amersham International and NEN. Other materials were of the highest grade available. Restriction fragments were sized against λ *Hin* dIII fragments, 1 kb ladder, and 123 bp ladder (all from BRL) on 1% agarose gels.

Nucleotide sequencing

Single-stranded phagemid DNA was prepared by M13KO7 superinfection of cultures of *E. coli* TG1 containing the appropriate phagemid according to Maniatis *et al.*[32], and was purified by FPLC on a Mono Q column[31]. Double-stranded DNA was prepared by the alkaline lysis procedure and purified by caesium chloride ultracentrifugation according to Maniatis *et al.*[32]. Single- and double-strand dideoxy sequencing was carried out using Sequenase 2.0 ^{R} according to manufacturer's recommendations[32]. Sequence information was read with an IBI gel reader and manipulated using MacVector ^{R} software.

Assay of expression in E.coli

Cells were pelleted at 4,000 rpm at 4°C for 15 min and resuspended in 50mM Tris.HCl, pH 8.0, containing lysozyme (1mgml^{-1}) (Sigma) and DNase I (10μgml^{-1}) (BRL) for 1 h. The cell debris was then removed by centrifugation at 13,500 rpm at 4°C for 20 min and the supernatant collected. Protein concentrations were measured by the method of Bradford[33]. SDS-PAGE was performed according to published procedures using molecular weight markers as described previously[16]. IPNS activity was assessed by the hole-plate stopped bioassay against *Staphyloccus aureus* NTCC 6571[34]. Activity was measured in International Units (I.U.) where 1 I.U. is defined as that amount of enzyme which produces 1 μmol of isopenicillin N/min.

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