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Production of *Streptomyces clavuligerus* isopenicillin N synthase in *Escherichia coli* using two-cistron expression systems

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SUMMARY

Streptomyces clavuligerus isopenicillin N synthase (IPNS) gene expression was achieved in *Escherichia coli* by the construction of two-cistron expression systems formed in the high copy number plasmid vector pUC119. These two-cistron constructions were composed of the IPNS gene and its flanking sequences which encoded an upstream open reading frame (ORF), the IPNS ribosome binding site and a putative transcription terminator. No *E. coli*- like *Streptomyces* promoter motif was present upstream of the IPNS gene therefore transcriptional regulation of the two-cistron system was provided by the *lac* promoter of pUC119. Enzymatically active IPNS was detected in *E. coli* cells harboring the recombinant plasmids thereby providing evidence for the activity of the IPNS ORF and for the feasibility of production of *S. clavuligerus* IPNS in *E. coli*. These results indicate that simple two-cistron constructions involving foreign gene flanking sequences may be used to express foreign proteins in *E. coli*.

INTRODUCTION

β-lactam compounds account for greater than 50% of the clinical use of antibiotics. The industrial scale production of penicillin and cephalosporin antibiotics has provided a cornerstone of biotechnology. Although these antibiotics are primarily derived from the eukaryotes *Penicillium chrysogenum* and *Cephalosporium acremonium*, prokaryotic β-lactam producers are also important since they possess greater biosynthetic diversity [20]. *Streptomyces clavuligerus*, which is the best characterized prokaryotic β-lactam producer, synthesizes β-lac-

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tam compounds of the penicillin (penicillin N), cephalosporin (desacetoxycephalosporin C), cephamycin (cephamycin C) and clavam (clavulanic acid) classes.

In an effort to develop strains of bacteria or fungi which produce novel β-lactam compounds, current research has been directed toward the genetic manipulation of the biosynthetic enzymes [44]. The initial work has focused on the enzyme isopenicillin N synthase (IPNS) since all cephalosporin and penicillin producers use this enzyme to catalyze the formation of the first β-lactam intermediate (isopenicillin N) via the oxidative cyclization of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) [20]. Consequently, the IPNS genes from *C. acremonium* [31], *P. chysogenum* [9], *Aspergillus nidulans* [29,42], *S. lipmanii* [42], *S. clavuligerus* [24] and *S. jumonjinensis* [37] have been isolated. However, the application of cloned IPNS genes in the production of novel antibiotics by whole cells or cell-free systems may require protein engineering to alter substrate specificity. To this end, it would be most useful to apply *E. coli*-based molecular genetics technology such as high level expression vectors, which could be used for the production of IPNS in quantities required for enzyme characterization by X-ray chrystallography, or site-directed mutagenesis [12]. However, a large number of factors have been identified which may limit or prevent the expression of heterologous genes in *E. coli* [17].

In this study, a 9.2 kb fragment of *S. clavuligerus* DNA previously shown to contain an ORF corresponding to IPNS [24] has been further characterized by restriction mapping, DNA sequence analysis and gene expression. This study establishes the feasibility of producing enzymatically active *S. clavuligerus* IPNS in *E. coli* and investigates the genetic elements which are required for IPNS production in *E. coli* using a two-cistron expression system. (A preliminary report of this work was presented at the Fourth American Society for Microbiology Conference on the Genetics and Molecular Biology of Industrially Important Microorganisms, Bloomington, IN, 2–7 October 1988.)

MATERIALS AND METHODS

Bacterial strains and plasmids

The plasmid pBL1, formed by the ligation of pUC119 [41] and a 9.2 kb fragment of *S. clavuligerus* NRRL, 3585 DNA, contains the *S. clavuligerus* IPNS gene [24]. The plasmid pUC119 was a gift from J. Vieira (Waksman Institute of Microbiology, Rutgers University). The plasmid pKT210 [1] was provided by K. Roy (University of Alberta). *E. coli* MC1022 (*lacΔ*M15) [10] was obtained from M. Mevarech (University of Tel Aviv). The RNase-deficient strain *E. coli* MRE600 was provided by W. Paranchych (University of Alberta). *E. coli* JM109 (*recA1*) [45] was obtained from Stratagene Corp. All *E. coli* strains were grown on LB medium [25] at 37°C unless otherwise specified. *S. clavuligerus* NRRL 3585 was grown as previously described [21].

DNA subcloning and restriction mapping

E. coli host strains were made competent and transformed by plasmid or bacteriophage recombinant DNA by the procedure of Morrison [27]. The large scale purification of plasmid DNA on CsCl gradients and the small scale preparation of plasmid DNA by an alkaline lysis procedure were conducted using standard methods [25]. Established techniques were also used for the construction of recombinant plasmids or bacteriophage [25]. Prior to subcloning, some DNA fragments were purified from preparative agarose gels by electroelution using an Elu-trap® apparatus (Schleicher and Schuell, Inc). Colonies of *E. coli* containing recombinant plasmids were identified by colony hybridization [16] or by Southern hybridization of digests of purified recombinant plasmid DNA immobilized on nylon membranes [30]. Hybridization was conducted under the stringent conditions previously described [24] using plasmid DNA probes prepared by nick-translation [25] or using recombinant M13 hybridization probes [19].

Detailed restriction mapping was conducted by

the partial digestion of end-labelled fragments as described by Smith and Birnsteil [38]. The position of each restriction site was confirmed by the analysis of single and double restriction enzyme digest patterns or by the examination of DNA sequence data using the MacGene® programs (Applied Genetic Technology, Inc).

In vitro transcription-translation

This was conducted according to published procedures [11].

Electrophoresis

Protein samples were analysed by SDS-polyacrylamide gel electrophoresis [6] and coomassie blue staining [15].

DNA sequence analysis

DNA sequencing was conducted according to the enzymatic, chain-terminating method of Sanger et al. [35] using overlapping subclones constructed in M13mp 18 and mp 19 [45] and propagated in *E. coli* strain JM109. Dideoxynucleotide sequencing reactions were conducted using the universal sequencing primer or the 17-mer 'dCATGAAACCCTCCTTGG' which hybridized to a region upstream of the IPNS gene. Gels containing 7 M urea and 40% formamide [26] were used to relieve the compressions in the banding pattern of DNA fragments in standard polyacrylamide gels [34]. All nucleotide sequencing reactions were repeated using the modified T7 DNA polymerase ('sequenase', United States Biochemical Corp.) and dITP in place of dGTP.

The nucleotide sequences obtained were analyzed for regions of dyad symmetry and RNA folding patterns using the DYAD and BIOFLD programs available from BioNet-Intelligenetics. The DNA sequences were also analyzed for large open reading frames using the computer program of Bibb et al. [4] which had been modified to operate on a MacIntosh Plus computer [40].

Expression of IPNS in E. coli

A suitable medium for the production of IPNS in *E. coli* MC1022 was 2YT medium (16 g Bacto-tryptone (Difco), 10 g yeast extract (Difco), 5 g NaCl/l) containing chloramphenicol (10 μg/ml) or ampicillin (35 μg/ml). *E. coli* cultures were incubated in Erlenmeyer flasks (liquid to flask volume ratio of 1:5) at 28°C and 280 rpm. Media which had been preincubated were inoculated to an OD (at 600 nm) of 0.05 with *E. coli* from an overnight culture. After 2 h incubation, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to some cultures to a final concentration of 0.2 mM. Following 1 to 5 h further incubation, the cultures were chilled on ice and the cells were harvested by centrifugation (2000 × *g*, 10 min, 4°C). The cell pellets were washed once, by centrifugation, using IPNS assay buffer (50 mM Tris-HCl (pH 7.5), 0.01 mM EDTA, 0.1 mM DTT), resuspended in 0.05 volumes of assay buffer and held on ice prior to sonication. The cells were broken by 2 × 30 s bursts from a 0.75 in diameter probe fitted to a Bronson Sonifer Cell Disrupter 350 set at an intensity level of 7. The cell lysates were frozen rapidly in a dry ice-ethanol bath and then stored at −75°C.

Purification and assay of IPNS

IPNS was partially purified from *E. coli* or *S. clavuligerus* cell lysates as previously described [21]. IPNS activity was assayed by HPLC [23] or bioassay [22]. One unit of IPNS activity was defined as the amount of enzyme which converts ACV to 1 μmol of isopenicillin N per min under the reaction conditions previously described [22]. Purified ACV and isopenicillin N were provided by Saul Wolfe (Queen's University, Kingston, Canada). Protein concentrations were determined by the method of Bradford [7] using γ-globulin as a standard.

RESULTS

Physical map of the IPNS gene containing fragment

A restriction map of the 9.2 kb *S. clavuligerus* DNA fragment of pBL1, which contains the IPNS gene, is presented in Fig. 1. This map is in agreement with previous data [24] but provides a more detailed restriction analysis. The 9.2 kb *S. clavuligerus* DNA fragment does not contain sites for the

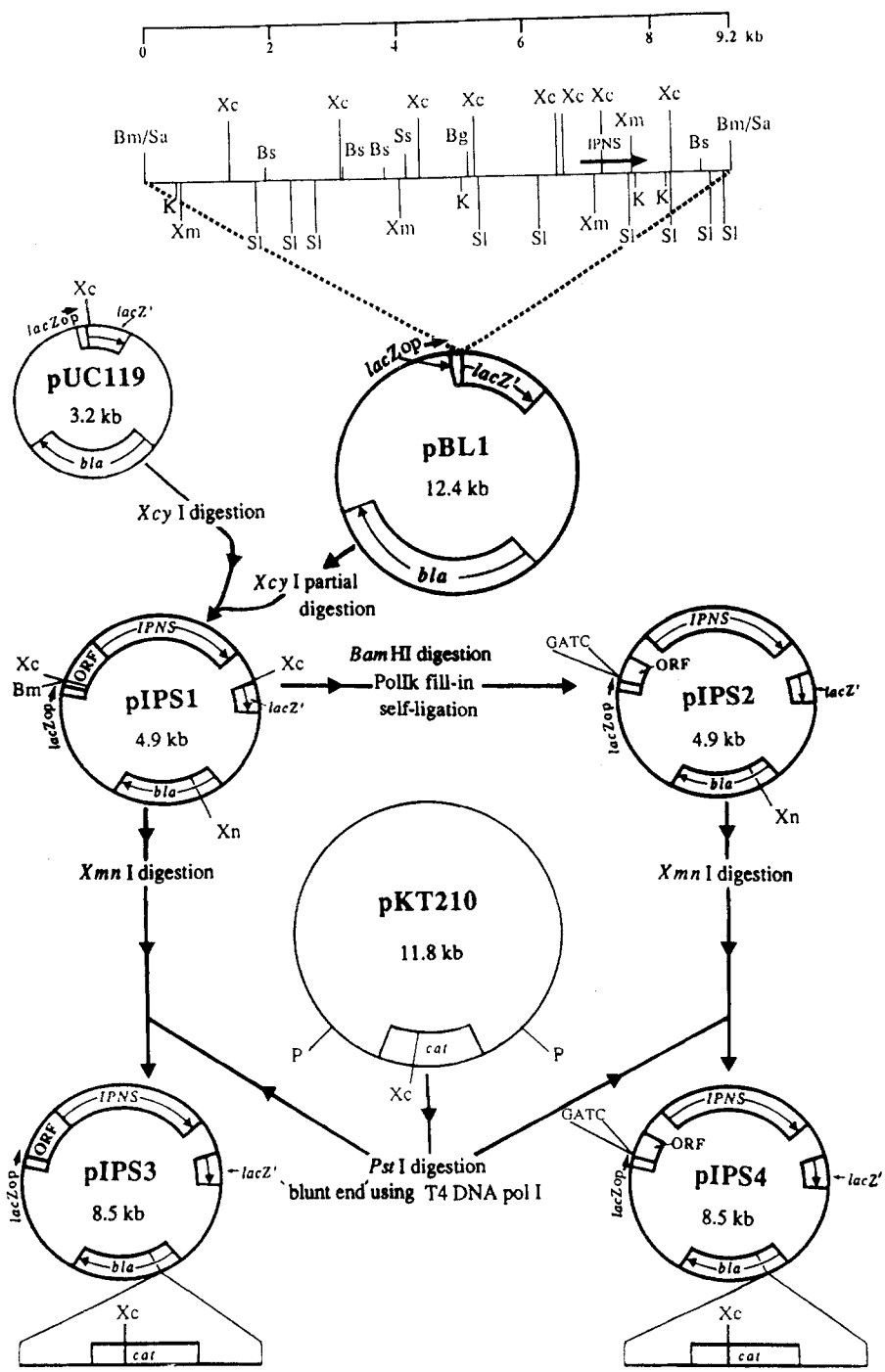

Fig. 1. Construction of IPNS expression vectors. A restriction map of the 9.2 kb *Streptomyces clavuligerus* DNA insert fragment of pBL1 is shown. The IPNS gene was isolated on a 1.7 kb *Xcy*I fragment and ligated into the *Xcy*I site within the multiple cloning site of pUC119 to create the plasmid pIPS1 (Ap^R). pIPS2 (Ap^R) was generated by *Bam*HI digestion of pIPS1 followed by the formation of blunt ends, by a 'fill-in' reaction, and self-ligation. The plasmids pIPS3 (Cm^R) and pIPS4 (Cm^R) were created by ligation of a 3.6 kb *Pst*I fragment containing the chloramphenicol acetyltransferase gene of pKT210 into the *Xmn*I site of pIPS1 and pIPS2, respectively. Further details concerning the molecular cloning are provided in the text. The plasmid constructions are not drawn to scale. The restriction map is drawn to the scale indicated directly above it. Abbreviations: Ap^r, ampicillin resistance; Bg, *Bgl*II; bla, β-lactamase; Bm, *Bam*HI; Bs, *Bst*EII; cat, chloramphenicol acetyltransferase; Cm^r, chloramphenicol resistance; *lacZ'*, the gene encoding the α-peptide of β-galactosidase; *lacZop*; the *lacZ* operator-promoter region; K, *Kpn*I; P, *Pst*I; PolIk, klenow fragment of *E. coli* DNA polymerase I; Sa, *Sau*3A; Sl, *Sal*I; Ss, *Sst*I; Xc, *Xcy*I; Xm, *Xma*I; Xn, *Xmn*I.

restriction enzymes *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I, *Nco*I or *Sph*I.

Nucleotide sequence of the IPNS gene flanking regions

Previously, pBL1 was shown to contain an ORF which corresponded in size and 5′ sequence to IPNS (MW 36.9 kDa) purified from *S. clavuligerus* [24]. pBL1 was shown to complement the IPNS-deficient strain *S. clavuligerus* NTG1, presumably via homologous recombination. Final evidence of the isolation of an active IPNS gene would involve expression of the IPNS ORF in a foreign host. Expression in *E. coli* was desirable since it would allow the subsequent manipulation of the IPNS gene using a variety of *E. coli*-based molecular genetics techniques.

No evidence for IPNS production was found in cell extracts of *E. coli* MC1022 harboring pBL1 or when pBL1 was introduced into an in vitro transcription-translation system prepared from *E. coli* MRE600 (data not shown). Therefore it was necessary to position the IPNS gene immediately downstream of an *E. coli* promoter. Examination of the sequence of the IPNS ORF [24] indicated a lack of useful restriction sites at the 5′ terminus of the IPNS gene, therefore it was determined to investigate the feasibility of construction of a two-cistron expression system in which *S. clavuligerus* DNA sequences flanking the IPNS ORF would be included. In order to characterize the flanking regions, the DNA sequence 709 bp upstream of the IPNS gene and 159 bp downstream of the IPNS gene was determined (Fig. 2).

The IPNS gene and its flanking regions were analyzed for evidence of typical *Streptomyces* structural gene coding sequences by examination of the G+C distribution as a function of codon position. The IPNS ORF, which has an overall G+C base composition of 67%, was recognizable by its preferential base frequency (Fig. 3). Calculations made directly from the DNA sequence indicated an average G+C bias at IPNS codon position 1 of 66%, at codon position 2 of 40%, and at codon position 3 of 97%. This same analysis indicated that another ORF, designated ORF1, existed immediately upstream of the IPNS ORF. Based on the predicted

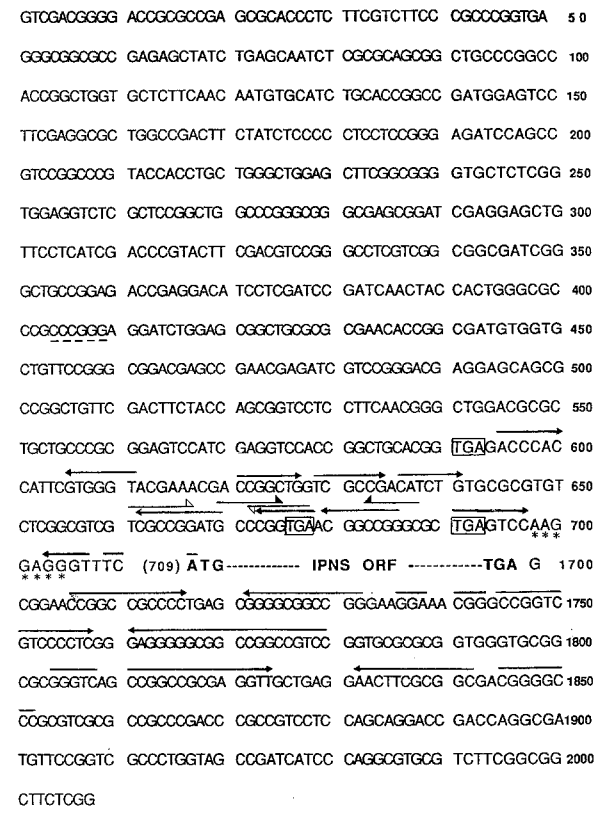

Fig. 2. Nucleotide sequences of *S. clavuligerus* IPNS gene flanking regions. The antisense strand has been represented. The IPNS coding sequence, which has been omitted, was published elsewhere [24]. Regions of dyad symmetry which would encode mRNA sequences capable of forming stem-loop structures have been indicated by overlying lines containing arrowheads or half-arrowheads. An *Xcy*I site located in the upstream flanking sequence (nts 404–409), which was used in the construction of the IPNS plasmid expression vectors, is underlined with a dashed line. The nucleotide sequence corresponding to the three translation termination codons ('TGA'; nts 591–593, 676–678, 691–693) located 16, 31 and 116 bp upstream of the IPNS gene, respectively, have been boxed. The nucleotides constituting the putative IPNS Shine-Dalgarno type sequence have been indicated with underlying asterisks.

protein sequence (data not shown) and the G+C bias illustrated in Fig. 3, ORF1 was considered to terminate at a translation stop codon ('UGA') located 31 bp upstream of the IPNS translation start codon ('AUG'; Fig. 2). A second upstream translation stop codon ('UGA') which is positioned in frame with this first one is located 16 bp upstream

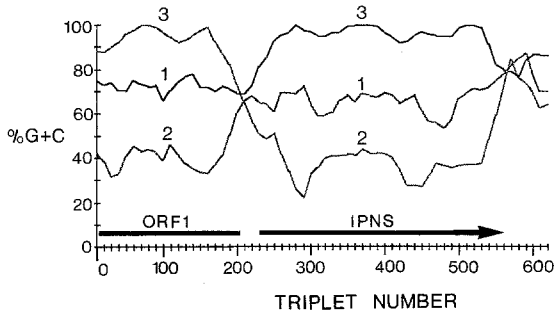

Fig. 3. G+C content as a function of codon position. DNA sequence data for the IPNS gene and flanking regions were analyzed for ORFs as described in Materials and Methods. The curves represent the G+C content at the codon positions indicated in the figure. The G+C content at each codon position was calculated using a window of 40 codons. The curves have been smoothed by averaging this data over each 10 data points which permits the representation of the curves using the MacIntosh Excel® graphics program. The relative position and orientation of the IPNS ORF is indicated by the arrow. The solid bar indicates the downstream region of another ORF, designated ORF1.

of the IPNS gene. No ORF was detected in the nucleotide sequence downstream of the IPNS ORF.

The IPNS flanking regions were examined for the presence of regulatory sequences. A nucleotide sequence 'AAGGAGG', which is complementary to a sequence near the 3′ end of the 16S rRNA of *S. lividans* [4], was located 5 bp upstream of the translation start codon for the IPNS gene. This sequence was considered to represent the Shine-Dalgarno type sequence within the IPNS ribosome binding site and was expected to support the efficient initiation of translation of the IPNS ORF in *E. coli* [18].

No *Streptomyces* promoter motifs [8,43], including *E. coli*-type *Streptomyces* promoters, were detected in the 31 bp intergenic region upstream of the IPNS ORF. However, this intergenic region does share 74% and 68% similarity with the nucleotide sequences immediately upstream of the IPNS genes of *S. lipmanii* [42] and *S. jumonjinensis* [37], respectively.

The nucleotide sequences downstream of the *S. clavuligerus* IPNS gene contained a series of G+C rich regions of dyad symmetry (Fig. 2) which would encode a RNA transcript capable of forming three consecutive, potentially highly stable, stem-loop structures. The first of these potential stem-loop structures was estimated to have a free energy of formation of approximately −39.3 Kcal/mol based on the calculations of Zuker and Stiegler [46]. This is characteristic of *Streptomyces* sequences which serve as effective transcription terminators in both *Streptomyces* spp. and *E. coli* [13]. No potentially highly stable stem-loop structures were encoded in the 31 bp intergenic region upstream of the IPNS gene, although small regions of dyad symmetry were evident (Fig. 2). The lack of a recognizable transcription terminator in the intergenic region suggests that an *E. coli* promoter located within ORF1 could direct transcription of the IPNS ORF.

Expression of the S. clavuligerus IPNS gene in E. coli

In order to express the IPNS gene in *E. coli*, transcription of the IPNS ORF was placed under the

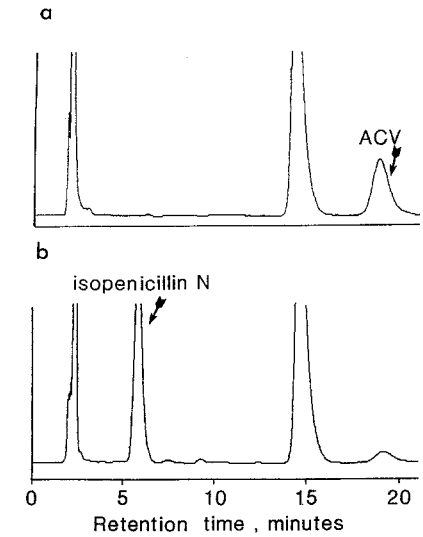

Fig. 4. HPLC analysis of IPNS activity. The HPLC analysis was conducted as described by Jensen et al. [23]. The reaction mixtures were incubated for 15 min prior to methanol deproteinization and application to the column. (a) A 'control' HPLC trace of the reaction mixture components incubated in the absence of additional proteins. (b) HPLC trace of the reaction mixture containing IPNS partially purified from *E. coli* (containing pIPS1) by the method used to purify IPNS from *S. clavuligerus* [21]. The elution position of ACV (the IPNS substrate) and isopenicillin N (the product of the enzyme reaction) are indicated.

control of the *lac* promoter of pUC119. This was accomplished by subcloning a 1680 bp *Xcy*I partial digest fragment containing the IPNS gene into the *Xcy*I site of pUC119 to create the plasmid pIPS1 (Fig. 1). This construction encoded a two-cistron system in which the IPNS ORF constituted the downstream ORF. The upstream ORF consisted of 267 bp from the 3′ end of *S. clavuligerus* ORF1 and 54 bp of the 5′ end of the ORF of the α-peptide of β-galactosidase (*lacZ′*). This gene fusion terminated 31 bp upstream of the *S. clavuligerus* IPNS ORF, therefore the IPNS ORF was involved in a transcriptional fusion but not a translational fusion. A modification of this two-cistron system was created by the introduction of 4 bp into the *Bam*HI site of pIPS1 (Fig. 1) which altered the reading frame involved in the upstream translational fusion. This new plasmid, designated pIPS2, encoded a translational fusion which would terminate at a 'UGA' codon located 116 bp upstream of the IPNS gene (Fig. 2).

Evidence for IPNS expression in *E. coli* MC1022 harboring either pIPS1 or pIPS2 was provided by HPLC analysis of enzyme activity in reaction mixtures containing crude cell lysates. IPNS production was evident only by the disappearance of the substrate, ACV (data not shown), since β-lactamase encoded by pIPS1 or pIPS2 destroyed isopenicillin N, the product of the enzyme reaction. Partial purification of IPNS from *E. coli* cell lysates allowed the demonstration of isopenicillin N formation in vitro (Fig. 4).

In order to enable the direct assay of isopenicillin N in cell lysates of *E. coli*, the β-lactamase genes of the plasmids pIPS1 and pIPS2 were insertionally inactivated using a 3.6 kb fragment of the plasmid pKT210 which contained a chloramphenicol acetyltransferase gene. These new IPNS expression vectors were designated pIPS3 and pIPS4, respectively (Fig. 1).

The results of assays of IPNS production directed by pIPS3 and pIPS4 are presented in Table 1. Cells of *E. coli* MC1022 containing pIPS3 were found to produce IPNS at a specific activity which was approximately equivalent to IPNS production by *S. clavuligerus* NRRL 8535 (0.0015 units per mg solu-

Table 1

Production of *S. clavuligerus* IPNS in *E. coli*

	IPNS Activity[a] (units/mg protein × 10^3)			
	pIPS3		pIPS4	
Culture Age[b] (hours)	+ IPTG	−IPTG	+ IPTG	−IPTG
1	0.17	0	0	0
2	0.63	0.06	0.07	0
3.5	1.48	0.24	0.28	0
5	1.19	0.91	0.31	0.27

[a] Cell extracts of *E. coli* MC1022 harboring IPNS expression vectors were assayed for IPNS activity by bioassay as described in Materials and Methods. One unit of IPNS activity was defined as the amount of enzyme which converted ACV to 1 μmol of isopenicillin N per minute under the reaction conditions previously described [22].

[b] Culture age refers to the incubation time after the addition of IPTG as described in Materials and Methods.

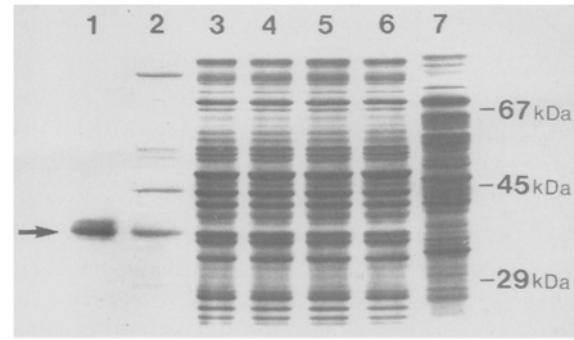

Fig. 5. SDS-PAGE of cell lysates of *E. coli* containing pIPS3 or pIPS4. The lanes contained the following samples: lane 1, IPNS purified from *S. clavuligerus*; lane 2, IPNS partially purified from IPTG induced *E. coli* containing pIPS3; lane 3, cell free extract of IPTG induced *E. coli* containing pIPS3; lane 4, cell free extract of uninduced *E. coli* containing pIPS3; lane 5, cell free extract of IPTG induced *E. coli* containing pIPS4; lane 6, cell free extract of uninduced *E. coli* containing pIPS4; lane 7, cell free extract of *S. clavuligerus*. Lanes 1 and 2 contained 7 μg of protein and lanes 3 to 7 contained 25 μg of protein. The proteins used as molecular weight standards included bovine serum albumin (67 kDA), ovalbumin (45 kDA) and carbonic anhydrase (29 kDa). The arrow indicates the position of IPNS on the gel.

ble cell protein). Comparison of IPNS gene expression directed by pIPS3 and pIPS4 showed that IPNS production was reduced 3 to 9 fold when translation of the upstream ORF was terminated 116 bp (pIPS4) rather than 31 bp (pIPS3) upstream of the IPNS ORF (Table 1). IPTG, an inducer of the *lac* promoter, was found to enhance IPNS production 6 to 10 fold at times soon after induction. No intracellular granules were observed by light microscopy in IPNS producing *E. coli* cells indicating that IPNS was accumulated in a soluble form.

E. coli cell extracts containing IPNS were analyzed by SDS-PAGE. No prominent band corresponding to IPNS was observed in any of the crude cell extracts (Fig. 5, lanes 3–6). This is not unexpected since no band corresponding to IPNS was detected in crude cell extracts of *Streptomyces clavuligerus* which contains IPNS at equivalent or greater specific activity (Fig. 5 lane 7). A protein of approximate MW 37,000, which co-migrated with IPNS purified from *S. clavuligerus*, was detected by SDS-PAGE following the partial purification of IPNS from *E. coli* MC1022 harboring pIPS3 (Fig. 5, lanes 1,2).

DISCUSSION

The expression of the *S. clavuligerus* IPNS ORF in *E. coli* provides final proof of the isolation of an active IPNS gene. IPNS produced in *E. coli* is enzymatically active, has the same apparent MW as *S. clavuligerus* IPNS, and may be purified from *E. coli* using the same protocol used to purify IPNS from *S. clavuligerus*. Therefore IPNS produced in either organism appears to be the same protein. Similarly, IPNS genes from three fungal species and the IPNS gene of *S. lipmanii* have been expressed in *E. coli* and the enzymes produced were indistinguishable from the native enzymes [3,9,29,42]. In each of these other cases, the IPNS ORF was cloned immediately downstream of an *E. coli* promoter. To accomplish this, the fortuitous existence of a restriction site, such as an *Nco*I or *Sph*I site located at the IPNS translation start site or the engineering of such a restriction site was required. However, most genes, including the *S. clavuligerus* IPNS gene, do not have useful restriction sites located at the 5′ terminus therefore it would be advantageous if a simple cloning strategy could be developed which utilized a restriction site located in the ORF upstream of the gene of interest. The result would be a two-cistron expression system.

Recently, a two-cistron system which encodes a synthetic upstream cistron designed to optimize the translation of eukaryotic proteins in *E. coli* [36] was used to direct the expression of the *C. acremonium* expandase/hydroxylase gene [33]. The two-cistron systems described in this study demonstrate the use of native sequences flanking the gene of interest in the expression of foreign genes in *E. coli*. This approach is feasible for genes which are not separated from an upstream ORF by a transcription terminator which is recognized in *E. coli*.

Knowledge of the DNA sequences flanking the IPNS ORF has permitted analysis of the three potential two-cistron IPNS expression systems which include an upstream translational fusion involving each of the three potential reading frames. It was found that there was no detectable IPNS production when a translational fusion was formed which is predicted to include the *lacZ′* gene, the IPNS ORF and the intervening region (data not shown). It was also found that when termination of translation of the fusion protein occurred 116 bp upstream of the IPNS ORF (pIPS4) rather than 31 bp upstream (pIPS3) there was a dramatic reduction in IPNS production. This reduction may be due to secondary structures formed in the region of mRNA encoding the IPNS Shine-Dalgarno sequence and the translation initiation codon (Fig. 2; predicted free energy of formation of -17.2 kcal/mol) which would inhibit the initiation of translation [18]. Enhanced IPNS production from pIPS3 would be explained by the phenomenon of translational coupling [18] in which a ribosome translating the upstream ORF disrupts this secondary structure thereby promoting the initiation of translation of the IPNS ORF. Alternatively, the differences in the activity of the pIPS3 and pIPS4 two-cistron expression systems may reflect differences in the levels of transcription of the IPNS gene since premature ter-

mination of translation, as occurs in the pIPS4 two-cistron expression system, has been observed to lead to a dissociation of RNA polymerase [39]. Clearly, optimal gene expression using two-cistron systems depends upon consideration of the DNA sequences flanking the gene of interest. The IPNS-ribosome binding site, which appears typical of prokaryotic ribosome binding sites [18], was recognized in *E. coli*. The large degree of secondary structure downstream of the IPNS ORF, recognized as a putative transcriptional terminator, also may have contributed to gene expression by inhibiting 3′ exonucleolytic degradation of the mRNA [28].

The *E. coli lac* promoter encoded by pUC119 was suitable for these studies since the moderate levels of gene expression obtained were sufficient to facilitate detection of IPNS activity without creating notable problems of toxicity which may be associated with the high-level production of foreign proteins in *E. coli* [17]. IPNS gene expression from the *lac* promoter was not strictly regulated by the addition of IPTG presumably because yeast extract contains inducing compounds [14] and because the *E. coli* host strain did not encode a $lacI^q$ gene.

It is anticipated that the pIPS3 two-cistron system will be amenable to the development of IPNS high level expression vectors utilizing *E. coli* promoters of high transcriptional activity. Production of large quantities of IPNS for X-ray crystallographic studies is a prerequisite for the rational site-specific mutagenesis of the *S. clavuligerus* IPNS gene required to alter substrate specificity [12]. Although similar studies of IPNS from *C. acremonium* are now being undertaken [32], studies of the *S. clavuligerus* IPNS may also be profitable since it shares with *C. acremonium* IPNS the ability to produce novel β-lactam antibiotics from substrate (ACV) analogs provided in cell-free systems [2,44].

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