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NOTES

High-Level Expression of the *Streptomyces clavuligerus* Isopenicillin N Synthase Gene in *Escherichia coli*

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The *pcbC* gene, which encodes isopenicillin N synthase (IPNS), was subcloned from *Streptomyces clavuligerus* into *Escherichia coli* by using the pT7 series of plasmid vectors. The polymerase chain reaction was used to introduce an *Nde*I site at the translation initiation codon of *pcbC*, allowing the gene to be inserted behind an *E. coli* type of ribosome binding site. This construction directed high-level expression of IPNS, but the IPNS was in an inactive form in inclusion bodies. Active IPNS was recovered by solubilizing and renaturing the protein.

β-Lactam compounds account for more than 50% of the world market for antibiotics and are produced by both prokaryotic and eukaryotic microorganisms. *Streptomyces clavuligerus* produces a variety of β-lactam compounds, including penicillin N, cephamycin C, and clavulanic acid. In view of its diverse biosynthetic capabilities, this species has become one of the best-characterized bacterial β-lactam producers (6). Isopenicillin N, the first β-lactam intermediate in the biosynthesis of penicillins and cephalosporins, is formed by the enzyme isopenicillin N synthase (IPNS) through oxidative cyclization of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (9). IPNS has been purified to homogeneity from *S. clavuligerus*, and the corresponding *pcbC* gene has been cloned and sequenced (8, 14).

Efforts aimed at increasing the specific activity and broadening the substrate specificity of IPNS to enable the enzyme to synthesize novel, unnatural antibiotics (24) depend on information about the active site of the enzyme. Biophysical techniques, such as X-ray crystallography, can provide information on the active site, but such analyses require larger amounts of purified protein than can be readily obtained from *S. clavuligerus* (8). Expression of *pcbC* in heterologous hosts may offer a more feasible means of obtaining these necessary amounts of IPNS. *pcbC* genes from *Streptomyces jumonjinensis* (13), *Streptomyces lipmanii* (23), *Aspergillus nidulans* (1, 18), *Cephalosporium acremonium* (1, 21), and *Penicillium chrysogenum* (1, 2) have been expressed at high levels in *Escherichia coli*. *pcbC* from *S. clavuligerus* has been expressed in *Streptomyces lividans* (7) and in *E. coli* by using a two-cistron expression system (3), but in each case the expression level was no greater than that seen in *S. clavuligerus*. Several factors can influence the level of expression of heterologous genes in *E. coli*, including transcription efficiency, translation initiation efficiency, and rare codon usage (4, 12). Heterologous genes, including *pcbC* genes from other *Streptomyces* species with codon usage quite different from that of *E. coli*, have been expressed at high levels in *E. coli*, suggesting that codon usage is not the main factor limiting the production of *S. clavuligerus* IPNS in the two-cistron expression system (1). Therefore, we attempted to express *pcbC* from *S. clavuligerus* by using the T7 expression system developed by Tabor and Richardson (22) to provide stronger transcriptional and translational control signals.

Plasmid expression vectors pT7-4, pT7-6, and pT7-7 contain the bacteriophage T7 promoter (ϕ10) located upstream of a multiple cloning site and also encode an ampicillin resistance gene (*bla*). In pT7-4, *bla* is oriented in the same direction as the T7 promoter and therefore expression of *bla* is influenced by the T7 promoter, whereas in pT7-6, *bla* is oriented opposite to the T7 promoter and expression is controlled solely by the *bla* promoter. pT7-7 contains the ribosome binding and translation initiation sites for the T7 ϕ10 protein located between the ϕ10 promoter and the multiple cloning site. The pT7 series of plasmids, helper plasmid pGP-1-2 (22), and *E. coli* K38 (19) were kindly provided by S. Tabor (Harvard Medical School, Boston, Mass.). pIJ2925, a derivative of *E. coli* plasmid pUC18 with an altered multiple cloning site flanked by *Bgl*II sites (15), was kindly provided by M. J. Bibb (John Innes Institute, Norwich, England). Standard procedures used for plasmid isolation and genetic manipulation of *E. coli* have been described by Sambrook et al. (20).

pcbC expression was carried out essentially as described by Tabor and Richardson (22). *E. coli* K38 cells carrying expression vector derivatives together with helper plasmid pGP-1-2 were inoculated into 400 ml of expression medium (2% Bacto Tryptone [Difco], 1% yeast extract [Difco], 0.5% NaCl, 0.2% glycerol, 50 mM potassium phosphate buffer [pH 7.2], 100 μg of ampicillin per ml, and 75 μg of kanamycin per ml) to an initial optical density at 600 nm of 0.05. The cultures were incubated at 30°C until the optical density at 600 nm was 1.5, transferred to 42°C for 30 min to induce production of T7 RNA polymerase, and then incubated at 37°C for an additional 2 h. The cultures were harvested by centrifugation, and the resulting cell pellets were washed with 200 ml of washing buffer (50 mM Tris-HCl [pH 7.5], 0.1 mM dithiothreitol, 0.01 mM EDTA, 10% glycerol) and resuspended in 8 ml of IPNS assay buffer (washing buffer

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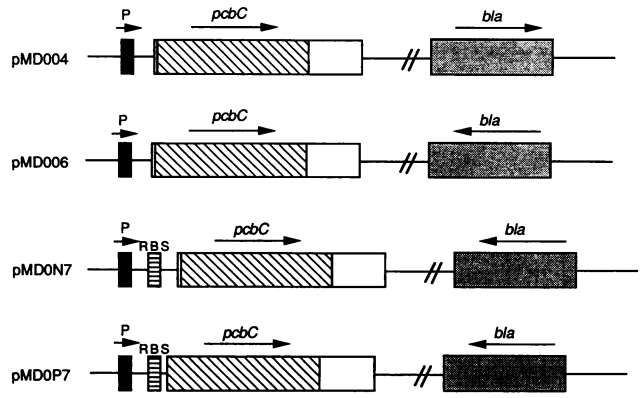

FIG. 1. pT7-derived *pcbC* expression vectors. The arrows indicate the orientations of the different structural elements. Abbreviations: P, T7-derived promoter for the ϕ10 gene; RBS, ribosome binding site for the T7 ϕ10 gene; *bla*, gene which encodes β-lactamase.

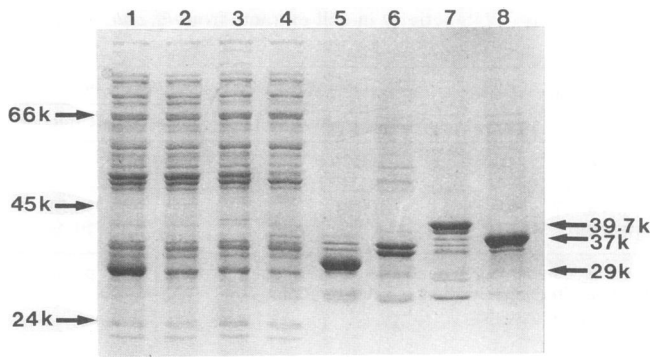

FIG. 2. SDS-PAGE analysis of cell extracts from *E. coli* carrying pMD004, pMD006, pMD0N7, and pMD0P7. The molecular weights of the marker proteins used are as follows: bovine serum albumin, 66,000; ovalbumin, 45,000; and trypsinogen, 24,000. Lanes 1 through 4 contained 30-μg portions of protein from the soluble fractions of cell extracts derived from cultures containing pMD004, pMD006, pMD0N7, and pMD0P7, respectively. Lanes 5 through 8 contained 15-μg portions of protein from the particulate fractions (after solubilization) of cell extracts derived from cultures containing pMD004, pMD006, pMD0N7, and pMD0P7, respectively. The arrow labeled 29k indicates the position of the β-lactamase protein band. The arrows labeled 37k and 39.7k indicate the positions of native and fusion IPNS proteins, respectively.

containing 1.0 mM dithiothreitol). The cells were disrupted by sonication and centrifuged at 17,000 × *g* for 10 min. The resulting pellet was resuspended in IPNS assay buffer, and the ultrasonic treatment and centrifugation were repeated twice. The final pellet was resuspended in 2 ml of IPNS assay buffer and then solubilized by adding 8 ml of a denaturing solution containing 6.25 M urea, 62.5 mM dithiothreitol, 1.25 mM EDTA, and 62.5 mM Tris-HCl (pH 7.5); the final protein concentration was 5 to 10 mg/ml. After 2 h at 21°C, the solution was desalted by gel filtration on a Sephadex G-25 column which was equilibrated and eluted in IPNS assay buffer. IPNS activity was assayed by a high-performance liquid chromatography procedure as described previously (10), using purified *bis*-δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, which was generously provided by S. Wolfe (Simon Fraser University, Burnaby, British Columbia, Canada).

In an attempt to achieve high-level expression of the *pcbC* gene, a fragment of *S. clavuligerus* DNA containing *pcbC* flanked by 335 bp of upstream sequence and 383 bp of downstream sequence was excised from plasmid pIPS-1 (3) by using *Eco*RI and *Hin*dIII and inserted into plasmid expression vector pT7-4. However, the resulting plasmid gave no detectable expression of IPNS protein (data not shown). A secondary-structure analysis of the nucleotide sequence upstream of the *pcbC* initiation codon showed that the putative transcript may form an imperfect stem-loop structure incorporating the ribosome binding site (RBS), which could limit translation initiation and prevent expression (17). This possible impediment to expression was eliminated by excising an *Xba*I-*Sty*I fragment, which removed all but 13 bp of the *S. clavuligerus* DNA sequence upstream of *pcbC* to generate pMD004 (Fig. 1). When *pcbC* expression was tested by using this construction, again no IPNS production was detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 2), indicating that some factor other than occlusion of the RBS by the secondary structure was preventing expression of *pcbC*. However, the presence of a prominent protein band at a molecular weight of 29,000 in the soluble fraction suggested that high-level expression of *bla* was occurring (Fig. 2). Since *pcbC* lies between *bla* and the T7 promoter, transcription of both *pcbC* and *bla* was presumed to be occurring, but only the *bla* region of the transcript was being translated efficiently. The 1.4-kb *pcbC*-containing insertion was excised from pMD004 as an *Eco*RI-*Hin*dIII fragment and was inserted into pT7-6 to give pMD006 (Fig. 1). In this construction, the *bla* gene is in the opposite orientation and is transcribed from its own promoter to give a low level of expression. The failure to detect *pcbC* expression in pMD006 (Fig. 2) excluded the possibility that the high level of expression of *bla* in pMD004 restricted *pcbC* expression and suggested that the sequence upstream of *pcbC* was not capable of supporting efficient translation initiation in *E. coli*.

An examination of the 13 bp of the *S. clavuligerus* DNA sequence upstream of *pcbC* in pMD004 revealed that the native RBS of *pcbC* matches the *E. coli* consensus Shine-Dalgarno sequence (AGGAGG) (3). However, the space between the RBS sequence and the initiator codon is only 5 bp, less than is optimal in *E. coli*, and the C residue immediately upstream of the initiation codon may have an additional detrimental effect on translation initiation (16). To examine the ability of the native *pcbC* RBS to initiate translation, the *pcbC*-containing *Eco*RI-*Hin*dIII fragment from pMD004 was cloned into pIJ2925 to acquire flanking *Bgl*II sites and then excised with *Bgl*II and inserted into the *Bam*HI site of pT7-7 to generate pMD0N7 (Fig. 1). This construction resulted in *pcbC* (with 13 bp of upstream sequence) being fused in frame to a fragment from the 5′ end of the T7 ϕ10 gene. An *E. coli* type of RBS precedes the fused gene, and within the fused gene the native *S. clavuligerus* RBS precedes *pcbC*. Therefore, transcription driven by the T7 promoter could give rise to either a fusion protein, authentic IPNS protein, or a mixture of the two. An examination of expression extracts by SDS-PAGE showed that high levels of the 39,700-Da fusion protein were produced, but no 37,000-Da IPNS protein was produced (Fig. 2). These results confirm that there is a strong preference for the *E. coli* translation initiation signals over the *pcbC* translation initiation signals. The native translation initiation

TABLE 1. IPNS activity in cell extracts from *E. coli* cultures carrying *pcbC* expression constructions

Expression construction	IPNS activity (U/mg of protein, 10^{-3})	
	Soluble fraction	Particulate fraction[a]
pMD004	ND[b]	ND
pMD006	ND	ND
pMD0N7	0.048	ND (30.21)
pMD0P7	0.718	ND (79.28)

[a] The numbers in parentheses are the values found in the particulate fraction after solubilization and renaturation.
[b] ND, not detected.

signals of the *S. clavuligerus pcbC* gene apparently are not able to support detectable levels of translation in *E. coli*.

The pMD0N7 construction directed high-level expression of the fusion protein, but for physicochemical analyses, authentic IPNS is required. The translation initiation codon of the T7 ϕ10 gene fragment within pT7-7 contains a unique *Nde*I site, and therefore creation of an *Nde*I site at the translation initiation codon of the *pcbC* gene should allow expression of authentic IPNS under the control signals optimized for *E. coli*. Introduction of an *Nde*I site required that three nucleotides, TTC, immediately preceding the wild-type *pcbC* gene be changed to CAT (shown underlined below). This alteration was achieved by the polymerase chain reaction (5), using TAGGAATTCCATATGCCAGT TCTGATGC and CGCCAGGGTTTTCCCAGTCACGAC (24-bp universal primer) as the oligonucleotide primers. Polymerase chain reaction mixtures contained 50 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 10 μg of bovine serum albumin, deoxynucleoside triphosphates (each at a concentration of 100 μM), 0.05% Tween 20, 5% dimethyl sulfoxide, 5 U of *Taq* DNA polymerase, 50 pmol of each primer, and 0.1 pmol of pIPS-1 (3) in a final reaction volume of 100 μl. The plasmid DNA was denatured for 3 min at 95°C, and 25 cycles of the polymerase chain reaction were conducted (each cycle consisted of 95°C for 0.5 min, 65°C for 1.0 min, and 72°C for 5.0 min); this was followed by a 10-min incubation at 72°C. DNA fragments amplified by the polymerase chain reaction were purified by using GeneClean (Bio 101, La Jolla, Calif.) and were cloned into plasmid vector pCR1000 (Invitrogen Corp., San Diego, Calif.) by using the conditions recommended by the supplier. A 1.4-kb DNA fragment containing the mutant *pcbC* gene with no upstream sequence was liberated by *Nde*I-*Eco*RI digestion and was inserted into the pT7-7 vector to generate pMD0P7 (Fig. 1). High-level expression resulted, and the protein expressed from this construction was solely in the form of authentic IPNS (Fig. 2). The IPNS protein was associated predominantly with the insoluble fraction of the cell extracts.

When cell extracts from the various pT7 constructions were assayed for IPNS activity, low levels of activity were observed in the soluble fractions of expression extracts from pMD0N7 and pMD0P7, and no activity was observed in the particulate fractions (Table 1). In contrast, SDS-PAGE of expression extracts had shown that the expressed protein was associated predominantly with the particulate fraction, and the intensity of the stained protein bands suggested that much larger amounts of IPNS protein were present than the activity measurements indicated. From this we concluded that the IPNS protein (or fusion protein) expressed from constructions pMD0N7 and pMD0P7 was predominantly in an inactive form in inclusion bodies, like numerous other homologous and heterologous recombinant proteins expressed at high levels in *E. coli* (11). Light and electron microscopy examinations of cells carrying pMD0N7 supported this conclusion (data not shown).

Active IPNS protein was recovered from the particulate fraction of expression extracts by disaggregation and solubilization of the insoluble material with urea under reducing conditions, followed by removal of the urea by gel filtration chromatography (Table 1). The specific activity of IPNS fusion protein in reactivated material from pMD0N7 was 30.21×10^{-3} U/mg of protein, while the specific activity of authentic IPNS from pMD0P7 extracts was 79.28×10^{-3} U/mg of protein. Assuming that the fusion protein has the same intrinsic IPNS activity as the authentic IPNS, this indicates that the pMD0P7 construction supports a higher level of expression than the pMD0N7 does, a conclusion which is also supported by the SDS-PAGE results. Although the majority of the IPNS protein is in an inactive particulate form, active protein can be generated by solubilization and refolding to yield a protein preparation with an IPNS specific activity that is 50-fold higher than that seen in cell extracts from *S. clavuligerus* (8). The inactive form of the recombinant IPNS makes it difficult to quantitate precisely the total amount of IPNS produced, but we estimate a yield of 50 mg of IPNS protein per liter of *E. coli* culture carrying pMD0P7.

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