Genetic Manipulation to Identify Limiting Steps and Develop Strategies for High-Level Expression of Penicillin Acylase in *Escherichia coli*

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Abstract: We have identified the bottleneck steps limiting expression of penicillin acylase (PAC) through comparison of the expression performance for various PAC-expression vectors constructed by genetically modulating the efficiencies of transcription and/or translation of the *pac* gene. To our knowledge, this is the first report demonstrating that expression of PAC could be limited by various steps, such as transcription, translation, and post-translational steps (i.e. translocation and periplasmic processing), depending on the host/vector systems. Results also indicate that the structure of the wild-type *pac* gene might not be optimal for direct use in production of PAC using recombinant DNA technology. To improve the gene expression, transcription was enhanced by manipulating certain DNA bases in the *pac* regulatory region, whereas translation was enhanced by enlarging the spacing between the ribosome binding site and the ATG initiation codon to increase the initiation efficiency. The information is useful in terms of developing genetic strategies for overproduction of recombinant PAC in *Escherichia coli.* © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **63:** 263–272, 1999.

Keywords: *Escherichia coli;* gene expression; genetic manipulation; penicillin acylase; recombinant DNA technology

INTRODUCTION

Penicillin acylase (PAC) from *Escherichia coli* is an enzyme which has a significant impact on the bulk pharmaceutical industry (Shewale et al., 1990; Shewale and Sivaraman, 1989). For protein maturation and developing the enzyme activity in the periplasm, the immediate translation product of the *pac* gene (preproPAC) has to be exported properly and then processed by periplasmic proteolysis, which is unusual for prokaryotic proteins (Fig. 1) (Sizmann et al., 1990). Although PAC has been applied in industry for decades and expression strategies based on recombinant DNA technology have been constantly developed, the production of the enzyme needs improvement. Previous works on production of PAC (Bhattacharya et al., 1993; Ramirez et al., 1994a, b; Robas et al., 1993; Sobotkova et al., 1995, 1996) critically tackled certain technical problems. The first was to develop proper host/vector systems with superior abilities on one or even several steps for protein synthesis (i.e., transcription and translation) and maturation (i.e., translocation and periplasmic processing). A second problem was to isolate mutants of PAC-superproducers (usually by random mutagenesis), perhaps followed by cloning the corresponding *pac* operon into a multicopy plasmid for overexpression.

As seen in Figure 1, all steps leading to mature PAC (i.e., transcription, translation, and post-translational steps) must be effective to efficiently produce the enzyme. However, the bottleneck step(s) limiting the gene expression should be identified before proper strategies are applied. Although transcription of the *pac* gene has not been experimentally shown to limit production of PAC up to now, a genetic strategy to improve the transcription (and perhaps, translation) efficiency by using a strong promoter system, such as *tac*, has been frequently adopted for enhancing the gene expression (Sriubolmas et al., 1997). Given a possible improvement in the PAC-expression level with this operation, formation of various PAC precursors without the enzyme activity in a soluble or insoluble form is not uncommon (Sriubolmas et al., 1997). It is known that synthesis of PAC in *E. coli* is usually induced by PAA (Vandamme, 1980) and catabolically repressed by glucose (Merino et al., 1992). However, genetic evidence with respect to these regulations is still limited (Valle et al., 1986), although the two carbons are likely to affect expression of the *pac* gene at a transcriptional level. On the other hand, information about translation of the *pac* gene is also rare and the majority is probably provided by a previous study (Keilmann et al., 1993) investigating the temperature dependency of translation. Due to the above limitations, it is a challenge to develop optimal expression strategies for the production of PAC.

In the current study, both transcription and translation efficiencies were genetically manipulated to identify limiting steps and develop strategies for high-level expression of

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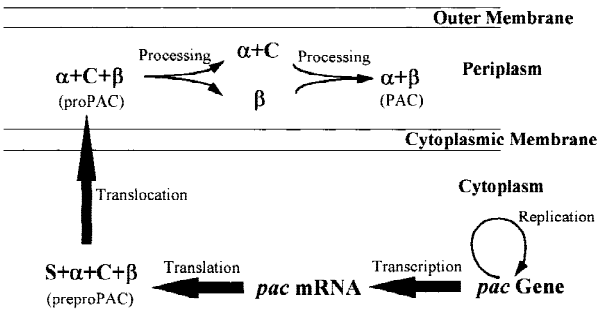

Figure 1. Synthesis and maturation of PAC in *E. coli*. The structural *pac* gene from *E. coli* ATCC11105 encodes a polypeptide precursor (prepro-PAC) which has a molecular weight of approximately 95 kDa and is composed of, in the direction of *N* to *C*, a signal peptide (S), an α subunit (α), a connecting peptide (C), and a β subunit (β). The signal peptide directs the export of preproPAC into the periplasm and is removed to form another type of precursor (proPAC) after the translocation. Periplasmic processing steps (i.e., proteolysis and folding) are followed to remove the connecting peptide and assemble the mature PAC (α+β).

PAC. Transcription efficiency was modulated through two approaches—either manipulating certain DNA bases in the *pac* regulatory region or using the *trc* promoter system. We also investigated the effects of translation efficiency which, to our knowledge, has not yet been studied for PAC. The idea originated from a possible translational limitation observed upon analyzing the DNA sequence near the ATG initiation codon of the wild-type *pac* gene (Oh et al., 1987). Translation in *E. coli* is usually limited by several factors, including initiation efficiency (Gualerzi and Pon, 1990), number of available ribosomes (Vind et al., 1993) and/or tRNAs (Smith, 1996), the secondary structure (de Smit and van Duin, 1990), and/or stability (Petersen, 1991) of mRNAs. Among them, initiation is usually considered as the major bottleneck step for translation (Gualerzi and Pon, 1990). The initiation efficiency is affected by the codons near the ATG initiation codon (Looman et al., 1987), the amino acid sequence of the ribosome binding site (Leipold and Dhurjati, 1993), and the spacing between the ribosome binding site and the ATG initiation codon (Stormo, 1986). Although the 'AAA' codon adjacent to the ATG initiation codon of the *pac* gene might pose some positive effects on the gene expression (Looman et al., 1987), the spacing at only four bases between the ribosome binding site and the ATG initiation codon tends to limit the efficiency of translation initiation (Stormo, 1986). By manipulating some of the above factors through conducting the site-directed mutagenesis experiments, translation efficiency could be modulated for identifying whether translation is a step-limiting expression of PAC. Several recent studies (Scherrer et al., 1994; Sriubolmas et al., 1997) indicated that expression of PAC is often limited by cytoplasmic or periplasmic aggregation of PAC precursors (i.e., by post-translational steps). We are demonstrating that, in addition to post-translational steps, transcription and translation could also become limiting for expression of PAC depending on the host/vector systems.

MATERIALS AND METHODS

Bacterial Strains and DNAs

Bacterial strains, plasmids, and oligonucleotides used in this study are summarized in Table I. Enzymes for DNA cloning were purchased from New England Biolabs (Beverly, MA, USA). Extraction of genomic DNAs or plasmid DNAs from *E. coli* strains, purification of PCR products, and extraction of DNA fragments from agarose gels were performed using appropriate commercial kits (QIAGEN, Valencia, CA, USA). Molecular cloning was performed according to standard protocols (Sambrook et al., 1989). DNA sequencing was performed in an autosequencer (ABI PRISM 377, Perkin Elmer, Foster City, CA, USA) using a dye-terminator cycle-sequencing ready reaction kit (Perkin Elmer).

Cloning of the *pac* Gene From ATCC11105

Two DNA fragments containing the *pac* gene from ATCC11105 at 2.9 kb and 3.2 kb, respectively, were PCR-amplified (Fig. 2a). The PCR was conducted at a volume of 100 L in an automated thermal cycler (THERMOLYNE, Amplitron® II, Dubuque, IA, USA). The 100-μL reaction mixture contained 20 nmole of each dNTP, 100 pmole of each primer, and 100 ng of ATCC11105 genomic DNA as the template. *Pfu* DNA polymerase (STRATAGENE, La Jolla, CA, USA) which has a proofreading activity at 0.025 unit/μL was used as the PCR polymerase to minimize the mutation frequency upon conducting the PCR experiments. The temperature profile was initiated with a hot start at 95°C for 4 min, followed by 25 cycles of a three-temperature profile (94°C for 1 min, 55°C for 2 min, and 72°C for 6.5 min), and terminated with a postdwell at 72°C for 7 min. Primer pairs of PC1/PC4 and PC3/PC4 (refer to Fig. 2a for the location of each primer) were used to amplify the corresponding 3.2-kb and 2.9-kb DNA fragments containing the *pac* gene. After the reaction, the PCR product was purified, digested with *Eco*RI/*Pst*I, and then ligated with the 3.6-kb *Eco*RI/*Pst*I-digested fragment of pBR322 to form the recombinant plasmids of pCLL3201 and pCLL2902 (Fig. 2b). DNA sequencing was performed to ensure that no mutation occurred in the regulatory region of the *pac* gene for both plasmids. The difference between the two plasmids is the extra 241 bps, which contain the putative CRP-binding sites and a possible operator for PAA-induction, in the *pac* regulatory region for pCLL3201 (Fig. 2a).

Site-Directed Mutagenesis

In vitro site-directed mutagenesis was conducted using the QuickChange™ kit (STRATAGENE) which allows site-specific mutations in a double-stranded plasmid. pCLL3201 and pCLL2902 were used as the templates, whereas oligonucleotide pairs of PM1/PM2 and PM3/PM4 were used as the mutagenesis primers. Various mutant plasmids were made by using appropriate templates and oligonucleotide pairs (Table I). All mutant plasmids constructed in this

Table I. List of bacterial strains, plasmids, and oligonucleotides in this study. The designed restriction sites in the oligonucleotides are underlined.

Strains and DNAs	Relevant genotype or phenotype	Source and Reference
E. coli		
ATCC11105	A PAC-producing strain derived from ATCC9637	CCRC[a]
GM48	F$^-$ *thr-l leuB6 dcm-6 dam-3 thi-l ara14 lacY1 galK2 galT22 tonA31 tsx-78 supE44* λ	CCRC (Marinus, 1973)
HB101	F$^-$ *hsdS20 leuB6 recA13 ara-14 proA2 lacY1 thi-l galK2 rpsL20 xyl-5 mtl-l supE44* λ$^-$	CCRC (Boyer and Roulland-Dussoix, 1969)
S. marcenscens		
ATCC27117	A penicillin-G-resistant and 6-APA-sensitive strain	ATCC
Plasmids		
pACYC177	A plasmid harboring the Km^R gene cassette, Ap^R, Km^R	CCRC (Chang and Cohen, 1978)
pBR322	A cloning vector, Ap^R, Tc^R	CCRC (Bolivar et al., 1977)
pBRAPS322	A control plasmid derived from pBR322 by self-ligating the largest *Dra*I fragment, Ap^S, Tc^R	This work
pCLL2902	A PAC expression vector containing the *pac* gene from ATCC11105 at 2.9 kb, Ap^S, Tc^R	This lab
pCLL3201	A PAC expression vector containing the *pac* gene from ATCC11105 at 3.2 kb, Ap^S, Tc^R	This lab
pTrc99A	A *trc*-expression vector, Ap^R	Egon Amann (Amann et al., 1988)
pTrcKn99A	A *trc*-expression vector derived from pTrc99A, Km^R	This work
pTrcKnPAC2902	An expression vector made by subcloning the *Eco*RI/*Pst*I *pac*-gene-containing fragment at 2.9 kb from pCLL2902 into pTrcKn99A, Km^R	This work
pTrcKnPAC3201	An expression vector made by subcloning the *Eco*RI/*Pst*I *pac*-gene-containing fragment at 3.2 kb from pCLL3201 into pTrcKn99A, Km^R	This work
pTrcKnPAC3211p	An expression vector made by subcloning the *Pst*I *pac*-gene-containing fragment at 2.8 kb from pYCC3211 into pTrcKn99A, Km^R	This work
pTrcKnPAC3221p	An expression vector made by subcloning the *Pst*I *pac*-gene-containing fragmemt at 2.8 kb from pYCC3221 into pTrcKn99A, Km^R	This work
pYCC2912	A PAC expression vector derived from pCLL2902 by site-direct mutagenesis using the primer pair of PM1/PM2, Ap^S, Tc^R	This work
pYCC2922	A PAC expression vector derived from pCLL2902 by site-directed mutagenesis using the primer pair of PM3/PM4, Ap^S, Tc^R	This work
pYCC3211	A PAC expression vector derived from pCLL3201 by site-directed mutagenesis using the primer pair of PM1/PM2, Ap^S, Tc^R	This work
PYCC3221	A PAC expression vector derived from pCLL3201 by site-directed mutagenesis using the primer pair of PM3/PM4, Ap^S, Tc^R	This work
Oligonucleotides		
PC1	5′GCAAATGCTGCCTGTCTGAATTCGAA 3′	This lab
PC3	5′ CTCACAGTTCATAATGAAAGAATTCCTCTG 3′	This lab
PC4	5′ CGGATAAACTGCAGCGTTACAAAGGG 3′	This lab
PM1	5′ CTAATTATACACCTGCAGGAGGATACAATG 3′	This work
PM2	5′ CATTGTATCCTCCTGCAGGTGTATAATTAG 3′	This work
PM3	5′ CTAATTATACACCTGCAGGAAAATACAATGAAAAATAG 3′	This work
PM4	5′ CTATTTTTCATTGTATTTTCCTGCAGGTGTATAATTAG 3′	This work

[a]Culture Collection & Research Center, Taiwan, ROC.

study could be easily screened by an extra *Pst*I site incorporated in front of the modified ribosome binding sites (described in Fig. 3).

Subcloning of the *pac* Gene

Because pTrc99A (Amann et al., 1988) contains the *bla* gene whose gene product of β-lactamase tends to attack the β-lactam bond of penicillin and, therefore, affect PAC enzyme assay, the *bla* gene was first replaced with a Km-resistance gene before subcloning of the *pac* gene (Fig. 4). To do this, pACYC177 was first amplified in GM48, which is a *dcm* mutant, to avoid the Dcm methylation at the two *Stu*I sites. The purified plasmid was cleaved with *Stu*I and the 1.3-kb DNA fragment containing the Km-resistance gene was gel-extracted. pTrc99A was digested with *Dra*I. The resulting largest DNA fragment was gel-extracted and ligated with the 1.3-kb Km-resistance cassette to form pTrcKn99A. The *Pst*I fragments, which contain the *pac* operon at about 2.8 kb, of pYCC3211 and pYCC3221 were

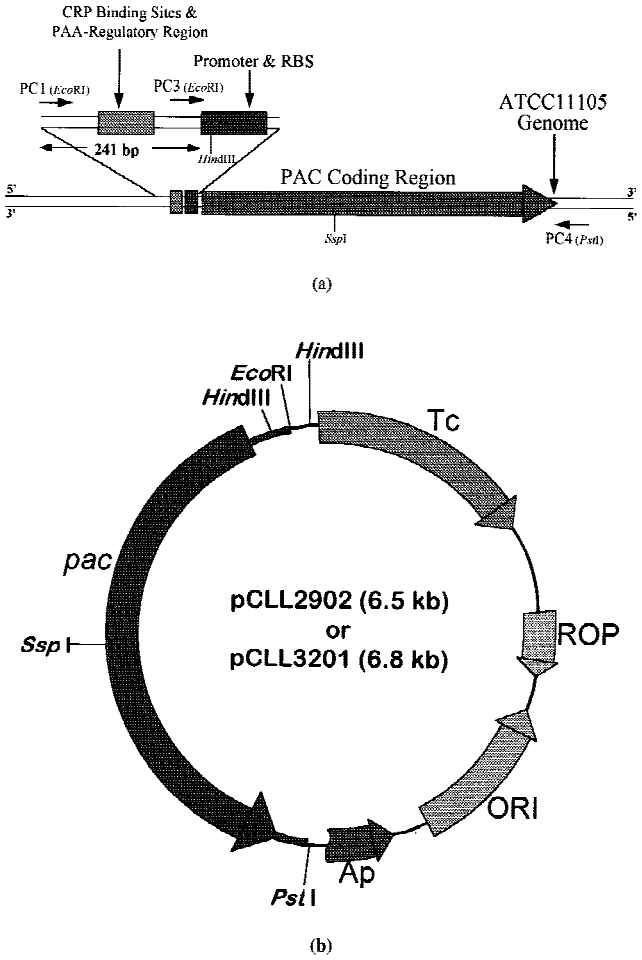

Figure 2. (a) The *pac* gene on the ATCC11105 genome and primer locations for PCR-amplification. The DNA size is 3.2 kb from PC1 to PC4 and 2.9 kb from PC3 to PC4. The restriction sites designed in the primers are parenthesized. (b) Restriction map of pCLL2902 and pCLL3201. The left-half *Eco*RI/*Pst*I fragment represents the cloned PCR product containing the *pac* gene (3.2 kb for pCLL3201 and 2.9 kb for pCLL2902), whereas the right-half one (3.6 kb) is from pBR322. The arrow of each gene indicates the direction of transcription.

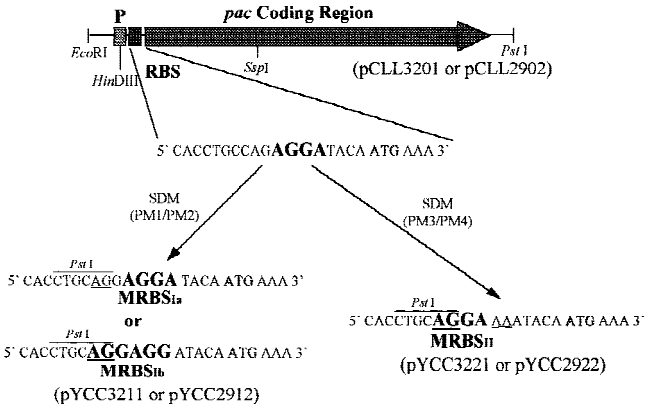

Figure 3. Modification of the ribosome binding site of the *pac* gene by site-directed mutagenesis (denoted as 'SDM'). The *Eco*RI/*Pst*I fragment which contains the wild-type *pac* gene from ATCC11105 in pCLL3201 or pCLL2902 is shown. The promoter (denoted as 'P'), ribosome binding site (denoted as 'RBS'), and coding region (with the arrow representing the direction of transcription) of the *pac* gene are also shown. DNA sequences near the ribosome binding site (enlarged and bold) before and after the site-directed mutagenesis are listed and the spacing between the ribosome binding site and the ATG initiation codon (shadowed), therefore, can be modulated. Mutagenesis was conducted using appropriate templates and primer pairs (names are parenthesized). DNA bases being mutated are underlined. Notice that an extra *Pst*I site was incorporated upon mutagenesis and could be used for not only screening the mutant plasmids (names are parenthesized) but also subcloning the mutated *pac* gene into pTrcKn99A to form transcriptional-fusion vectors. See texts in Materials and Methods for detailed experimental procedures.

subcloned into pTrcKn99A to form pTrcKnPAC3211p and pTrcKnPAC3221p, respectively. To do this, pTrcKn99A was first digested with *Pst*I, then dephosphorylated with alkaline phosphatase (CIAP), and finally ligated with the above *Pst*I fragments. The recombinant plasmids were subjected to restriction analysis for ensuring the proper orientation of the subcloned *pac* gene with respect to the *trc* promoter. In both pTrcKnPAC3211p and pTrcKnPAC3221p, the native *pac* promoter and CRP binding sites of the subcloned *pac* gene were deleted, therefore transcription of the *pac* gene was controlled by the *trc* promoter. The modified ribosome binding sites created in pYCC3211 and pYCC3221 were still available in pTrcKnPAC3211p and pTrcKnPAC3221p, respectively, for translation. To make pTrcKnPAC2902 and pTrcKnPAC3201, the *Eco*RI/*Pst*I fragments from pCLL2902 (at 2.9 kb) and pCLL3201 (at 3.2 kb), respectively, were subcloned into pTrcKn99A.

Microbiological Screening of PAC-Producing Strains

PAC-producing strains can be identified with a microbiological screening protocol which is an overlaying test using a bacterial strain, *Serratia marcenscens* ATCC27117, resistant to penicillin but sensitive to 6-APA (Oostendorp, 1972).

Batch Cultivation

Escherichia coli cells were cultivated in MPAC (0.5% NaCl, 0.5% Bacto beef extract, and 1% Bacto yeast extract) or LB (0.5% NaCl, 0.5% Bacto yeast extract, and 1% Bacto tryptone) medium. If necessary, PAA (at 0.1%) or IPTG (at 0.05 m*M*) was supplemented for inducing synthesis of PAC. Antibiotics at the following concentrations in mg/L were used: Ap, 50; Tc, 10; and Km, 25. Cells were initiated by streaking the −80°C glycerol-containing stock culture on an LB agar plate. The plate was incubated at 37°C for about 12 h. An isolated single colony was picked to inoculate MPAC medium at 5 mL in a 15-mL vial, which was then shaken at 220 rpm and 37°C in a rotary shaker for about 12 h. The culture at about 4 mL was used as the inoculum. Batch cultivations using 50 mL MPAC-based medium in 250-mL Erlenmeyer flasks were conducted in a reciprocating shaker at 200 rpm and 28°C for about 20 h.

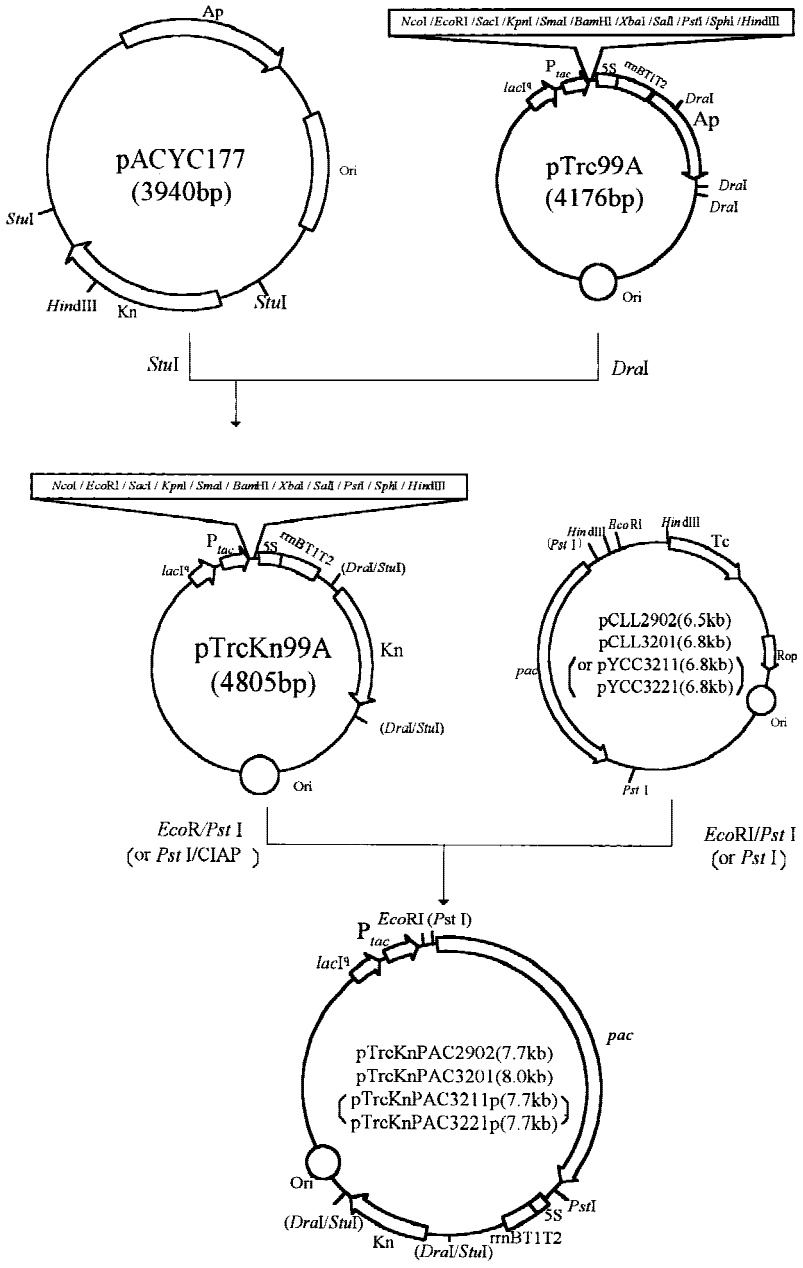

Figure 4. Schematic diagram of constructing pTrcKn-plasmids for expression of PAC using the *trc* promoter. The extra *Pst*I site (parenthesized) exists only for pYCC-plasmids, pTrcKnPAC3211p, and pTrcKnPAC3221p. See texts in Materials and Methods for detailed cloning procedures.

Treatment of Samples

Cell culture samples at 10 mL were centrifuged at 2°C and 6000*g* for 5 min. The cell pellets were resuspended in 2 mL sodium phosphate buffer (0.05*M*, pH 7.5). The cell suspensions were sonicated for 2 min using an ultrasonic processor (SONICS & MATERIALS, Danbury, CT, USA) and then centrifuged at 2°C and 16,000*g* for 15 min. The supernatants containing soluble proteins were assayed for PAC enzyme activities. The pellets containing insoluble proteins were resuspended in TE/SDS buffer (10 m*M* Tris HCl, pH 8.0, 1 m*M* EDTA, 1% SDS) and heated at 100°C for 5 min. Protein contents of the pellets (as insoluble fractions) and the supernatants (as soluble fractions) were analyzed by SDS-PAGE and Western blotting.

PAC Enzyme Assay

PAC enzyme was assayed at 37°C using penicillin G as a substrate (Gang and Shaikh, 1976). The amount of enzymatic reaction product of 6-APA was quantified using a colorimetric method developed previously (Balasingham et al., 1972). One unit was defined as the amount of enzyme hydrolyzing 1.0 μmole penicillin G per min at 37°C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A 14% polyacrylamide separating gel stacked by a 4% polyacrylamide stacking gel was prepared in a Mini-

PROTEAN®II electrophoresis cell (BIO-RAD, Hercules, CA, USA) according to manufacturer's instructions. Protein samples and a mixture of standard protein markers (FMC, Rockland, ME, USA) were loaded for analysis. Electrophoresis was conducted under a constant voltage of 200 volts for at least 30 min. After electrophoresis, the gel was stained with Coomassie blue and was scanned.

Immunological Analysis (Western Blotting)

After SDS-PAGE, proteins on the polyacrylamide gel were electroblotted to a PVDF membrane using a transfer electrophoresis unit (HOEFER, TE 22, San Francisco, CA, USA) according to a standard protocol (Towbin et al., 1979). Electrophoresis was conducted at a constant current of 0.4 amp for 1 h. Protein-antibody hybridization was performed as described by Sambrook et al. (1989). The primary antibody against PAC was raised in a rabbit intermittently immunized with purified PAC (SIGMA, St. Louis, MO, USA). After several weeks, the antiserum was collected from the immunized rabbits and was treated with HB101 lysate to remove anti-*E. coli* antibodies as described by Sambrook et al. (1989). The secondary antibody was goat anti-rabbit IgG conjugated with HRP. Mature PAC and PAC precursors were detected by a colorimetric method using DAB as a substrate and the processed membranes were scanned.

RESULTS AND DISCUSSION

Genetically Modulating Transcription and/or Translation Efficiencies

To demonstrate the effects of transcription or translation efficiency on expression of PAC, appropriate expression systems should be used because overexpression of PAC tends to form inclusion bodies (Scherrer et al., 1994; Sriubolmas et al., 1997) and complicate the characterization results. Expression plasmids of pCLL2902 and pCLL3201 were used in this study. Transcription of the *pac* gene was controlled by the native *pac* promoter for both plasmids. Due to the difference in the *pac* regulatory region (Fig. 2), expression of PAC was induced by PAA for pCLL3201 and was constitutive for pCLL2902 (Chou et al., 1999). The two plasmids gave relatively high PAC expression levels by which investigation on the effects of transcription or translation was possible. More importantly, PAC precursors at minimal amounts accumulated in cells (Fig. 5; see later discussion), suggesting that expression of PAC for the two plasmids was likely limited by transcription or translation instead of post-translational steps.

Effects of transcription efficiency on expression of PAC were characterized through the use of a strong *trc* promoter system. pTrc99A (Amann et al., 1988) was used in this study for several reasons. First, it contains the *trc* promoter, whose strength is about the same as that of the *tac* promoter, and the $lacI^q$ gene required for regulation. Second, because pTrc99A is derived from pBR322, all expression vectors constructed in this study are expected to have about the same copy number based on which the gene dosage effects on expression of PAC could be eliminated. Before subcloning the *pac* gene into the *trc* expression vector, the Ap-resistance marker in pTrc99A was replaced by a Km-resistance cassette to form pTrcKn99A (Fig. 4) so that the attack on the β-lactam bond of penicillin by the *bla* gene product of β-lactamase could be avoided. Various transcriptional-fusion vectors (pTrcKnPAC-plasmids) for expression of PAC were constructed by subcloning DNA fragments containing the *pac* gene at different sizes into the multiple cloning site of pTrcKn99A (Table I). In addition to the use of the *trc* promoter, transcription efficiency could also be enhanced by modulating certain DNA bases in the *pac* regulatory region (discussed in the next sections).

We genetically manipulated the *pac* ribosome binding site by site-directed mutagenesis. Effects of translation efficiency on expression of PAC were investigated by designing two types of modifications on the *pac* ribosome binding site (Fig. 3). pYCC2912 and pYCC3211 were derived from pCLL2902 and pCLL3201, respectively, through the first modification using the oligonucleotide pair of PM1/PM2 as the mutagenesis primers. Similarly, pYCC2922 and pYCC3221 were derived from pCLL2902 and pCLL3201, respectively, through the second modification using the oligonucleotide pair of PM3/PM4 as the mutagenesis primers. By comparing the PAC expression levels of the mutant (pYCC-) plasmids with those of the corresponding parent (pCLL-) plasmids, it appears that the first modification of the ribosome-binding site (referred as $MRBS_I$) impaired the translation whereas the second one (referred as $MRBS_{II}$) enhanced it (Table II). Upon analyzing the first modification, there are two alternative binding patterns (Fig. 3) which could confuse ribosome binding to the *pac* mRNA and therefore, interfere with translation initiation. In addition, the spacing between the modified ribosome binding site and the ATG initiation codon was less than five bases, which were detrimental to translation initiation, for both binding patterns. On the other hand, not only was the above confusion for ribosome binding cleared, but also the DNA spacing was enlarged to be seven bases through the second modification (Fig. 3). Based on the results, the two modifications could be presumed as genetic manipulations for either decreasing (for the first type) or increasing (for the second type) the translation efficiency of the *pac* gene.

Effects of Transcription and/or Translation Efficiencies on Expression of PAC

By comparing the expression performance of the parent plasmid, pCLL2902, and the mutant plasmids, pYCC2912 and pYCC2922, it appears that expression of PAC for pCLL2902 was likely limited by translation. The results also explain why the specific PAC activity for pCLL2902 was significantly higher than that for pCLL3201. We believe this was primarily caused by efficient transcription for pCLL2902 based on the following arguments. First, the *pac*

Table II. Expression performance for various PAC expression vectors.

PAC expression vector	Type of regulatory loci: Promoter	Type of regulatory loci: Ribosome binding site	PAC expression performance: Cell density (OD_{600})[a]	PAC expression performance: Specific PAC activity ($U/L/OD_{600}$)[a]	PAC expression performance: Formation of PAC inclusion bodies
pCLL2902	*pac*	NRBS[b]	7.3 ± 0.3[c]	56.3 ± 11.8[c]	None
pYCC2912	*pac*	$MRBS_I$	6.4 ± 0.5[c]	29.0 ± 4.2[c]	None
pYCC2922	*pac*	$MRBS_{II}$	7.5 ± 1.1[c]	84.0 ± 14.0[c]	None
pCLL3201	*pac*	NRBS[b]	7.0 ± 0.1[d]	13.6 ± 0.9[d]	None
pYCC3211	*pac*	$MRBS_I$	6.1 ± 0.2[d]	11.8 ± 0.7[d]	Few
pYCC3221	*pac*	$MRBS_{II}$	7.4 ± 0.4[d]	16.3 ± 2.7[d]	None
pTrcKnPAC2902	*trc/pac*[f]	NRBS[b]	6.4 ± 1.1[e]	38.9 ± 8.3[e]	None
			5.4 ± 1.0[g]	63.1 ± 4.2[g]	Some
pTrcKnPAC3201	*trc/pac*[f]	NRBS[b]	5.7 ± 0.2[e]	11.7 ± 2.9[e]	None
			5.9 ± 0.1[g]	25.8 ± 1.3[g]	Few
pTrcKnPAC3211p	*trc*	$MRBS_I$	5.3 ± 0.2[e]	2.8 ± 0.9[e]	None
			5.5 ± 0.1[g]	21.3 ± 4.3[g]	Some
pTrcKnPAC3221p	*trc*	$MRBS_{II}$	4.8 ± 0.1[e]	12.1 ± 0.9[e]	None
			2.1 ± 0.1[g]	14.3 ± 1.7[g]	Many

[a]Results are averages of three identical cultivations. See texts for description of batch cultivation.
[b]Native *pac* ribosome-binding site.
[c]Without PAA-induction.
[d]With PAA-induction at 0.1%.
[e]Without IPTG-induction.
[f]The native *pac* promoter is included in the subcloned *pac* operon.
[g]With IPTG-induction at 0.05 m*M*.

mRNAs synthesized based on either of the two plasmids (pCLL2902 and pCLL3201) should be identical because transcription of the *pac* gene for both plasmids was controlled by the native *pac* promoter. These *pac* mRNAs harbored the same native *pac* ribosome-binding site resulting in the same translation efficiency. Second, no PAC precursors accumulated in cells either in a soluble form or as inclusion bodies (Fig. 5), suggesting that the expression was not limited by any of the post-translation steps for the two plasmids. Third, the difference between the two plasmids is the 241 bps in the *pac* regulatory region, which is responsible for several carbon-related regulations, such as glucose catabolite repression and PAA-induction (Valle et al., 1986). Because regulations of these types usually appear at a transcriptional level, it is plausible to correlate the low PAC activity for pCLL3201 with inefficient transcription caused by the *pac* regulatory region. The correlation was elucidated by expression of the *pac* gene devoid of this regulatory region using pCLL2902. Not only was the expression no longer subjected to PAA-induction and glucose catabolite repression (Chou et al., 1999), but the specific PAC activity for pCLL2902 was also much higher than that for pCLL3201 (Table II). It was then presumed that the extra 241 bps in the *pac* regulatory region for pCLL3201 somehow hindered transcription of the *pac* gene. In that case, expression of PAC for pCLL3201 was likely limited by transcription. Deletion of these DNA bases for pCLL2902 highly improved the transcription efficiency, therefore the limiting step could shift to translation. The argument of transcriptional limitation for pCLL3201 was further supported by results of modifying the *pac* ribosome binding site in pCLL3201. The specific PAC activities for the mutant plasmids of pYCC3211 and pYCC3221, which respectively harbored the modified ribosome binding sites of $MRBS_I$ and $MRBS_{II}$, were comparable to that for the parent plasmid of pCLL3201 (Table II). The results suggest that expression of PAC for pCLL3201 was minimally affected by modulating the translation efficiency.

Other evidence demonstrating the effects of translation efficiency on expression of PAC was provided based on the expression performance for pTrcKnPAC3211p and pTrcKnPAC3221p. In pTrcKnPAC3221p, transcription and translation of the *pac* gene were simultaneously enhanced by the *trc* promoter and $MRBS_{II}$. However, the specific PAC activity was very low and PAC precursors at a tremendous amount accumulated in cells upon IPTG-induction (Fig. 6). Cell growth was severely inhibited (Table II) possibly due to the toxicity from these *pac* gene products. The results indicate that the limiting step for expression of PAC shifted to one of the post-translational steps depending on the location of PAC precursors. In pTrcKnPAC3211p, transcription was enhanced by the *trc* promoter whereas translation was impaired due to the use of $MRBS_I$ for ribosome binding. The amount of PAC precursors was significantly reduced (Fig. 6), and cell growth was no longer inhibited (Table II) upon IPTG-induction. The specific PAC activity slightly increased compared to pTrcKnPAC3221p, but still remained low possibly due to inefficient translation. Although PAC precursors in the insoluble fractions could be detected by Western blotting for the two *trc*-constructs (Fig. 6c), only those for pTrcKnPAC3221p were visible on the SDS-PAGE gel (Fig. 6b), indicating that the amount of PAC inclusion bodies for pTrcKnPAC3221p was significantly larger than that for pTrcKnPAC3211p. The amount of PAC

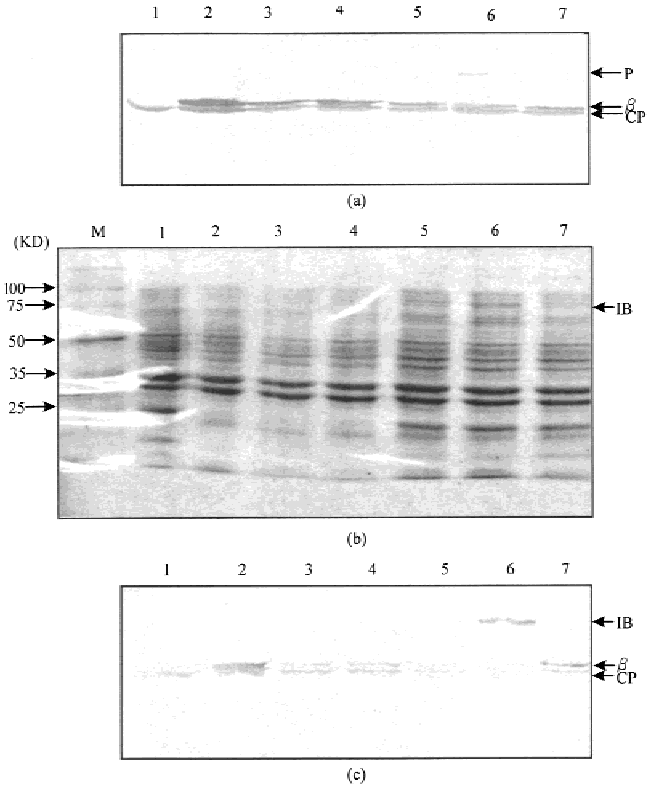

Figure 5. Results of SDS-PAGE and immunological analysis on protein contents of soluble (a) and insoluble (b and c) fractions for various cell lysates. HB101 was the host for expression of PAC. pBRAPS322, made by self-ligating the largest *Dra*I fragment at 3.65 kb from pBR322, was used as a control plasmid. Lane M/ markers; lane 1/ pBRAPS322; lane 2/ pYCC2922; lane 3/ pYCC2912; lane 4/ pCLL2902; lane 5/ pYCC3221; lane 6/ pYCC3211; lane 7/ pCLL3201; CP/ a contaminated *E. coli* protein existing in the purified PAC (SIGMA) which was used as the antigen for raising anti-PAC antibodies; IB/ PAC precursors existing in cells as inclusion bodies; P/ soluble PAC precursors; β/β subunit.

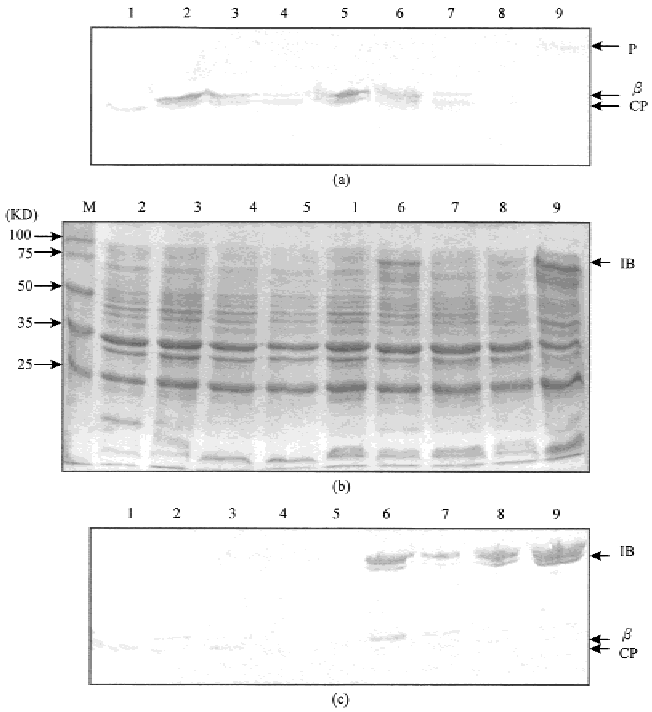

Figure 6. Results of SDS-PAGE and immunological analysis on protein contents of soluble (a) and insoluble (b and c) fractions for various cell lysates. HB101 was the host for expression of PAC. pTrcKn99A was used as a control plasmid. Lanes 2–5 are lysate samples from uninduced cultures and lanes 6–9 are those from IPTG-induced cultures. Lane M/ markers; lane 1/ pTrcKn99A; lanes 2 and 6/ pTrcKnPAC2902; lanes 3 and 7/ pTrcKnPAC3201; lanes 4 and 8/ pTrcKnPAC3211p; lanes 5 and 9/ pTrcKnPAC3221p; CP, IB, P, and β/ same as those in Figure 5.

precursors in the soluble fraction for pTrcKnPAC3221p was also larger than that for pTrcKnPAC3211p (Fig.6a). The results indirectly suggest that translation of the *pac* gene for pTrcKnPAC3221p was more efficient than that for pTrcKnPAC3211p.

Strategies for High-Level Expression of PAC

pCLL3201 contains the complete wild-type *pac* gene (including the regulatory and coding regions) from ATCC11105. It appears that inefficient transcription and translation tend to limit high-level expression of PAC using recombinant DNA technology. Because transcription was identified as the limiting step for expression of PAC with pCLL3201, enhancing the transcription efficiency turned out to be an appropriate strategy for improving the yield. Usually, expression of recombinant proteins could be significantly improved by using a strong promoter system, such as *tac* or T7 (Goeddel, 1990). Other types of problems, however, are subject to arise. For example, it is common that overexpressed polypeptides by using a strong promoter system would aggregate in host cells as inclusion bodies without the enzyme activity (Strandberg and Enfors, 1991). The problem is even severe for the case of overexpressing proteins destined for being exported. The pre-exported polypeptides or immature precursors tend to form insoluble inclusion bodies in the cytoplasm or even periplasm (Bowden et al., 1991; Sriubolmas et al., 1997). In this study, deletion of the above-mentioned 241 bps in the *pac* regulatory region (pCLL2902) or the use of the *trc* promoter for controling the transcription (pTrcKnPAC3201) significantly improved expression of PAC (both compared to pCLL3201; Table II) due to enhanced transcription. The limiting step shifted to translation for pCLL2902 based on the following observations. Further increasing the transcription efficiency by using the *trc* promoter (pTrcKnPAC2902) did not significantly improve the expression, whereas increasing the translation efficiency by using a modified ribosome binding site of $MRBS_{II}$ (pYCC2922) highly improved the expression (both compared to pCLL2902; Table II). Therefore, pYCC2922 could be treated as an improved version of pCLL3201 through a simultaneous enhancement on transcription and translation of the *pac* gene. Another plasmid of pTrcKnPAC3221p could also be treated as, similar to pYCC2922, an improved version of pCLL3201. However, the specific PAC activity was low due to the post-translational limitation caused by saturation of the machin-

ery for protein export or periplasmic processing. Therefore, using a strong promoter such as *tac*, *trc*, or T7 to enhance transcription of the *pac* gene might not be suitable for overexpression of PAC. A minimal PAC activity was also observed in our lab by using the T7 promoter system for regulation (unpublished data).

CONCLUSIONS

PAC provides an ideal model system demonstrating that heterologous expression of recombinant proteins in *E. coli* could be limited by various steps including transcription, translation, and post-translational steps. Factors limiting expression of PAC were identified in the current study. First, transcription of the *pac* gene was inefficient due to certain DNA bases in the *pac* regulatory region. Second, translation of the *pac* mRNA was limited by a short spacing at only four bases between the ribosome-binding site and the ATG codon. Third, the protein export and/or periplasmic processing machinery tended to be overwhelmed by various PAC precursors. The limitations could be overcome by developing genetic strategies toward high-level expression of PAC. In essence, a balanced flux throughout the protein synthesis (i.e., transcription and translation) and maturation steps (i.e., translocation and periplasmic processing) should be appropriately maintained to avoid potential accumulation of immature PAC precursors and optimize the *pac* gene expression.

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NOMENCLATURE

Ap	ampicillin
bps	base pairs
DAB	3,3′-diaminobenzidine tetrahydrochloride
HRP	horseradish peroxidase
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilo base pairs
Km	kanamycin
PAA	phenyl acetic acid
PAC	penicillin acylase
PCR	polymerase chain reaction

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