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Chaperone-Mediated Folding and Maturation of the Penicillin Acylase Precursor in the Cytoplasm of *Escherichia coli*

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Expression of the leaderless *pac* gene (LL *pac*), which lacks the coding region for the signal peptide of penicillin acylase (PAC), in *Escherichia coli* was conducted. It was demonstrated that the PAC precursor, proPAC, can be produced and even processed to form mature PAC in the cytoplasm, indicating that the posttranslational processing steps for PAC maturation can occur in both the periplasm and the cytoplasm of *E. coli*. The outcome of proPAC folding and PAC maturation could be affected by several factors, such as inducer type, proPAC formation rate, and chaperone availability. Misfolding of proPAC in the cytoplasm could be partially resolved through the coexpression of cytoplasmic chaperones, such as trigger factor, GroEL/ES, or DnaK/J-GrpE. The three chaperones tested showed different extents of the effect on proPAC solublization and PAC maturation, and trigger factor had the most prominent one. However, the chaperone-mediated solublization of proPAC did not guarantee its maturation, which is usually limited by the first autoproteolytic step. It was observed that arabinose could act as an effective inducer for the induction of LL *pac* expression regulated by the *lac*-derived promoter system of *trc*. In addition, PAC maturation could be highly facilitated by arabinose supplementation and coexpression of trigger factor, suggesting that the coordination of chaperone systems with proper culture conditions could dramatically impact recombinant protein production. This study suggests that folding/misfolding of proPAC could be a major step limiting the overproduction of PAC in *E. coli* and that the problem could be resolved through the search for appropriate chaperones for coexpression. It also demonstrates the analogy in the issues of proPAC misfolding as well as the expression bottleneck occurring in the cytoplasm (i.e., LL *pac* expression) and those occurring in the periplasm (i.e., wild-type *pac* expression).

Although proteins of interest can be overproduced in various hosts through successful applications of recombinant DNA technology, *Escherichia coli* remains the most common host system as a protein overproducer for industrial applications. Biochemical and genetic engineering strategies have been developed toward high-level gene expression and high-cell-density cultivation using *E. coli* (1, 3, 20). Among many technical problems, the formation of intracellular insoluble protein aggregates, known as inclusion bodies, is a common factor limiting the overproduction of gene products (4). While the mechanism of inclusion body formation is not completely understood, models and pathways regarding protein folding in the cells have been proposed (10, 33). A possible reason for inclusion body formation is that the overexpressed gene products cannot be suitably processed by folding modulators to generate a correct protein structure (4, 30). For extracytoplasmic proteins, the efficiencies for translocation, postexport folding, processing, and targeting become important as well. In principle, the precursors, intermediates, or final gene products can possibly form inclusion bodies in the cytoplasm and/or periplasm upon gene overexpression. This brings up a technical issue that, in addition to improving the efficiency of each gene expression step (i.e., transcription, translation, and posttranslational steps), a "balanced" protein synthesis flux throughout these steps should be properly maintained to avoid the accumulation of any protein species along the protein formation pathway.

Using penicillin acylase (PAC), an important industrial enzyme for the production of many β-lactam antibiotics (12, 27), as the target protein, strategies for enhancing recombinant protein production in *E. coli* have been developed in our lab (7, 8, 19, 23). Formation of mature PAC in the periplasm of *E. coli* involves a series of posttranslational steps, including translocation and periplasmic processing/folding steps, which are unique for prokaryotic proteins (Fig. 1A) (28). The periplasmic processing mechanism consists of various proteolytic steps, including the intramolecular autoproteolysis for the first cleavage of the PAC precursor (proPAC; at the junction between the connecting peptide and β subunit) and the subsequent proteolyses for chopping the connecting peptide (18, 28). The formation of inclusion bodies, which are primarily composed of proPAC in the periplasm, was recently identified as an important obstacle for the overproduction of PAC in *E. coli* (8, 26, 29).

We previously demonstrated that the presence of exogenous DegP significantly reduced the amount of periplasmic proPAC inclusion bodies and enhanced the production of recombinant PAC in *E. coli* (19, 23). Though the protease activity of DegP was shown to be primarily linked with the improvement (23), the possible contribution of the DegP chaperone activity could not be completely excluded. The results suggested that DegP

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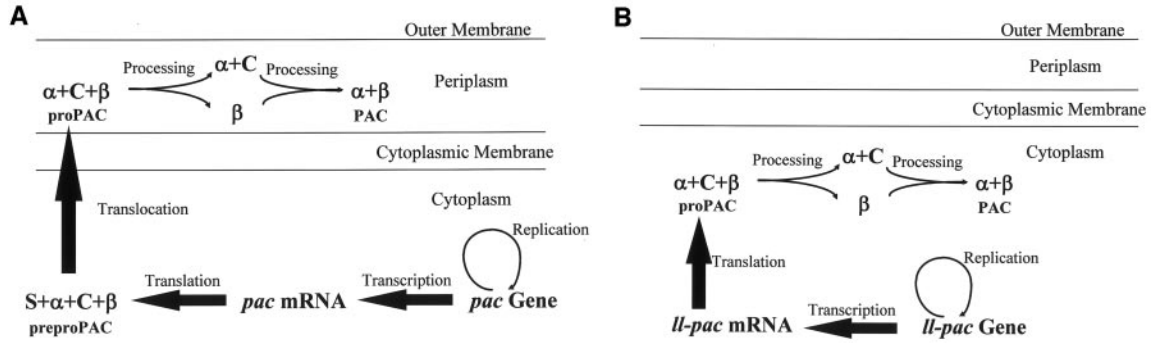

FIG. 1. PAC formation pathway based on the expression of wild-type *pac* (A) and LL *pac* (*ll-pac*) (B) in *E. coli*. (A) The structural wild-type *pac* gene encodes a polypeptide precursor (preproPAC) composed of, starting at the N terminus, a signal peptide (S), an α subunit (α), a connecting peptide (C), and a β subunit (β). The signal peptide directs the export of preproPAC into the periplasm and is cleaved after translocation. Another type of PAC precursor, proPAC, is formed in the periplasm. The periplasmic processing (i.e., PAC maturation) involves a series of proteolytic steps, resulting in the removal of the connecting peptide and the assembly of two subunits (α and β) to form active PAC. (B) The structural LL *pac* gene encodes the prePAC precursor, which is subjected to PAC maturation in the cytoplasm without being exported (see the text).

could suppress the physiological toxicity in at least two ways (23). First, it degraded or refolded various abnormal periplasmic proteins generated upon *pac* overexpression. Second, it interacted with proPAC directly to prevent its misfolding or even to recover (or degrade) the misfolded proPAC. It was proposed that an appropriate folding status of proPAC should be attained for the subsequent periplasmic processing and that such a folding process could possibly be assisted by chaperones (23). Therefore, the search for appropriate folding modulators that improve proPAC folding would provide valuable hints for identification of the key factor limiting the production of recombinant PAC.

The current study was conducted specifically to address this issue. Through the construction of the expression plasmid containing the *pac* gene devoid of the coding region for the signal peptide (i.e., leaderless [LL] *pac*), proPAC was expressed in *E. coli*. The expressed proPAC could remain in soluble or insoluble form or even be processed for PAC maturation to form active PAC in the cytoplasm of *E. coli* (Fig. 1B). A selection of cytoplasmic chaperones, including trigger factor, GroEL/ES, and DnaK/J-GrpE, was coexpressed for investigating their effect on in vivo folding and processing of proPAC. The analogy in the issues of proPAC misfolding and expression bottleneck occurring in the cytoplasm (i.e., LL *pac* expression) and those occurring in the periplasm (i.e., wild-type *pac* expression) was demonstrated.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains, plasmids, and oligonucleotides used in this study are summarized in Table 1 and are briefly described below. JM109 (32) was used as the host for LL *pac* expression. Molecular cloning was performed according to standard protocols (25) using HB101 (6) as the cloning host. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). PCR was performed in an automated thermal cycler (Amplitron II; Thermolyne, Dubuque, IA). Plasmid DNA was purified using a spin column kit purchased from BD Biosciences Clontech (Palo Alto, CA). Plasmid transformation was carried out according to the method of Chung and Miller (9) or using an electroporator (*E. coli* Pulser; Bio-Rad, Hercules, CA).

The plasmids containing the cytoplasmic chaperone gene(s) were derivatives of pAR3 (24) with a chloramphenicol-resistant (Cm^r) marker. pG-KJE8 (22) contains the *dnaK/J::grpE* and *groEL/ES* operons, respectively, fused with the *araB* and *zt-lp* promoters. pG-Tf3 (22) contains the *tig* gene (encoding trigger factor) and *groEL/ES* operon, respectively, fused with the *araB* and *zt-lp* promoters. pTrcKn29S contains the leaderless *pac* structural gene (LL *pac*), encod-

TABLE 1. Strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Relevant genotype/phenotype or sequence[a]	Source (reference)[b]
E. coli strains		
HB101	F^- *hsdS20 leuB6 recA13 ara-14 proA2 lacY1 thi-1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	CCRC (6)
JM109	F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]/*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1 mcrA* Δ(*lac-proAB*)	CCRC (32)
Plasmids		
pG-KJE8	P_{araB}::*dnaK/J*::*grpE* $P_{zt\text{-}lp}$::*groEL/ES*, Ori (pACYC184), Cm^r	T. Yura (22)
pG-Tf3	P_{araB}::*tig*, $P_{zt\text{-}lp}$::*groEL/ES*, Ori (pACYC184), Cm^r	T. Yura (22)
pTrcKn99A	A P_{trc} expression vector derived from pTrc99A, Ori (pBR322), Kn^r	This study (8)
pTrcKn29S	P_{trc}::LL *pac*, Ori (pBR322), Kn^r	This study
Oligonucleotides[c]		
PE1	5′-GCACTGGCTGA***ATTC***TCGTCAAG-3′	This study
PE2	5′-CATATTCCT*G*CAGAATATGAGGGCT-3′	

[a] Designed restriction sites are underlined and introduced mutations are in boldface.
[b] CCRC, Culture Collection & Research Center, Taiwan.
[c] Primer pair for amplification of LL *pac*.

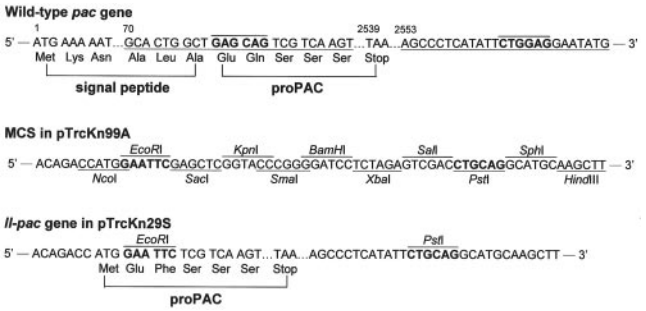

FIG. 2. Construction of pTrcKn29S. The DNA region from nucleotides 79 to 2570 of the wild-type *pac* gene, with appropriate mutations for generating the restriction sites (boldface), was cloned into the EcoRI and PstI sites (boldface) of pTrcKn99A to form pTrcKn29S for LL *pac* expression. The regions of the DNA sequences corresponding to the primers (PE1 and PE2) for PCR amplification are underscored in the wild-type *pac* gene. MCS, multiple cloning sites.

ing proPAC (i.e., α+C+β), fused with the *trc* promoter (Fig. 2). To construct pTrcKn29S, the DNA fragment corresponding to the region of proPAC was PCR amplified with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using the primer pair PE1 and PE2 and the template pCLL2902 (8). The 2.5-kb PCR product flanked with EcoRI and PstI was purified and cloned into the EcoRI and PstI restriction sites of pTrcKn99A (8), resulting in the formation of the in-frame *pac* gene without the signal peptide. pTrcKn29S has a pBR322 replication origin and a kanamycin-resistant (Kn^r) marker and is therefore compatible with various chaperone-gene-containing plasmids with a pACYC184 replication origin.

Cultivation. Cells were revived by streaking the stock culture stored at −80°C on a Luria-Bertani (LB) agar plate (5 g/liter NaCl, 5 g/liter Bacto yeast extract, 10 g/liter Bacto tryptone, 15 g/liter Bacto agar). The plate was incubated at 37°C for approximately 15 h. An isolated single colony was picked to inoculate 25 ml of LB medium, which was then incubated at 37°C in a rotary shaker at 200 rpm for approximately 15 h. The medium was supplemented with 50 μg/ml kanamycin or 34 μg/ml chloramphenicol when necessary. Erlenmeyer flasks containing 25 ml LB medium were inoculated with the seed culture and were shaken in a rotary shaker at 28°C and 200 rpm. When the cell density reached an optical density at 600 nm (OD_{600}) of approximately 0.5, the culture was supplemented with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG), 5 mg/ml arabinose, and/or 20 ng/ml tetracycline to induce the expression of the gene regulated by the *trc*, *araB*, and *zt-lp* promoters, respectively. After induction, the Erlenmeyer flasks were further shaken under the same conditions for another 4 h. All cultivations were conducted at least in duplicate.

Analytical methods. The culture sample was appropriately diluted with saline solution for measuring cell density at OD_{600} with a spectrophotometer (DU520; Beckman Coulter, Fullerton, CA). For the preparation of cell extract, cells at 20 OD_{600} units (defined as $OD_{600} \times$ ml) were centrifuged at 2°C and 6,000 × *g* for 10 min. The cell pellet was resuspended in 0.75 ml of sodium phosphate buffer (0.05 M, pH 7.5). The cell suspension was sonicated for 2 min using an ultrasonic processor (Misonix, Farmingdale, NY) and then centrifuged at 2°C and 15,000 × *g* for 15 min. The supernatant containing soluble proteins was assayed for intracellular PAC activity. The pellet containing insoluble proteins and cell debris was washed with phosphate buffer, resuspended in TE-SDS buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA, 1% sodium dodecyl sulfate) and heated to 100°C for 5 min. The protein content of the pellet was analyzed as the insoluble fraction.

PAC-producing strains can be identified with a microbiological screening protocol which is an overlaying test using a bacterial strain, *Serratia marcescens* ATCC 27117, which is resistant to penicillin but sensitive to 6-aminopenicillanic acid (6-APA) (21). PAC was assayed at 37°C using penicillin G as a substrate (14). The amount of enzymatic reaction product of 6-APA was quantified using a colorimetric method developed previously (2). All assays were conducted in duplicate. One unit was defined as the amount of enzyme that hydrolyzed 1.0 μmol penicillin G per min at 37°C. The volumetric activity (in U/liter) is the product of the specific activity (in U/liter/OD_{600}) and cell density (in OD_{600}).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-PROTEANII electrophoresis cell (Bio-Rad, Hercules, CA) using a 15% polyacrylamide separating gel stacked with a 4% polyacrylamide stacking gel. Protein samples of the cell extract from cells of 0.08 OD_{600} units and 0.5 OD_{600} units for the soluble and insoluble fractions, respectively, were loaded for SDS-PAGE analysis (0.2 and 1.0 OD_{600} units for the soluble and insoluble fractions, respectively, were used for Western blotting analysis). Electrophoresis was conducted under a constant voltage of 60 V for approximately 5 h. The gel was stained with Coomassie blue and dried in a hood. The dried gel was then scanned.

To conduct Western blotting, after SDS-PAGE, the proteins on the polyacrylamide gel were electroblotted to a polyvinylidene difluoride membrane using a Mini Trans-Blot cell (Bio-Rad, Hercules, CA) according to a standard protocol (31). The electrophoretic transfer was conducted at a constant voltage of 100 V for 1 h. Protein-antibody hybridization was performed as described by Sambrook et al. (25). The primary antibodies against the heterodimer and the β subunit of PAC, respectively, were raised in a rabbit intermittently immunized with the corresponding antigens. PAC was originally purchased from Sigma (St. Louis, MO). Prior to immunization, the PAC heterodimer was purified by anion-exchange and hydroxyapatite chromatography using a low-pressure chromatographic system (BioLogic LP; Bio-Rad, Hercules, CA), whereas the β subunit was prepared by elution of the protein band from polyacrylamide gel slices using an Electro-Eluter (model 422; Bio-Rad, Hercules, CA). The secondary antibody was goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Sigma, St. Louis, MO). PAC-related polypeptides were detected by a colorimetric method using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the substrate. The processed membrane was scanned.

RESULTS

Expression of LL *pac*. Figure 2 shows the construction of pTrcKn29S by cloning of the LL *pac* gene encoding proPAC, and the expression of LL *pac* was under the regulation of the *trc* promoter. With such a design, the first 2 amino acids of Glu-Gln in the N terminus of proPAC were replaced by the tripeptide sequence Met-Glu-Phe. The clone was first investigated qualitatively by conducting the microbiological overlaying test using a bacterial strain, *Serratia marcescens* ATCC 27117, which was resistant to penicillin but sensitive to 6-APA (21). A clear growth inhibition zone corresponding to the pTrcKn29S-harboring strain was observed, indicating that PAC activity was detected. The result implies that proPAC can be properly expressed and processed to form mature PAC in

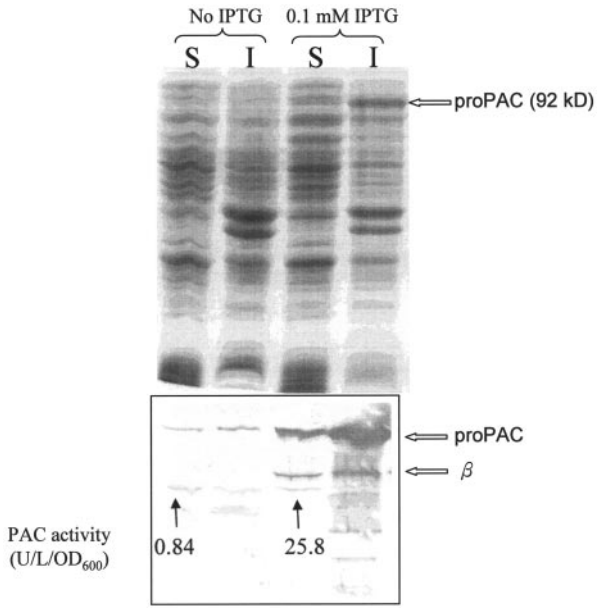

FIG. 3. SDS-PAGE and Western blotting analysis of the soluble (S) and insoluble (I) protein fractions of the culture samples of JM109 (pTrcKn29S) with and without IPTG induction.

TABLE 2. Expression of LL *pac* for JM109 (pTrcKn29S, pG-KJE8) under various culture conditions[a]

Culture supplement or parameter	Result for culture:						
	A-1	A-2	A-3	A-4	A-5	A-6	A-7
IPTG	−	+	+	+	+	−	−
Tetracycline	−	−	+	−	+	+	−
Arabinose	−	−	−	+	+	−	+
Cell density (OD_{600})	2.4 ± 0.0	2.3 ± 0.0	2.5 ± 0.1	2.3 ± 0.1	2.7 ± 0.1	2.5 ± 0.0	2.2 ± 0.1
Sp act (U/liter/OD_{600})	3 ± 1	45 ± 4	32 ± 11	112 ± 11	79 ± 8	3 ± 2	100 ± 2

[a] Induction of LL *pac*, *groEL/ES*, and *dnaK/J-grpE* was supposedly effected by the supplementation of IPTG, tetracycline, and arabinose, respectively. +, inducer was added; −, inducer was not used.

the cytoplasm of *E. coli*. In addition, the change in the N-terminal amino acid sequence appeared to be harmless to the processing of proPAC and the activity of mature PAC. Using JM109 (pTrcKn29S) as a host/vector system, LL *pac* overexpression was performed in shake flasks with LB-based medium. The formation of both proPAC and mature PAC was induced when the culture was supplemented with 0.1 mM IPTG. Part of the induced proPAC remained in the insoluble fraction (Fig. 3). On the other hand, PAC activity increased 30-fold (25.8 U/liter/OD_{600} for the induced culture versus 0.84 U/liter/OD_{600} for the noninduced control) without growth inhibition (2.18 OD_{600} for the induced culture versus 2.19 OD_{600} for the control). As the formation of insoluble proPAC suggested the existence of a protein-folding issue for this expression system, the effect of several cytoplasmic chaperones, including trigger factor, GroEL/ES, and DnaK/J-GrpE, on LL *pac* expression was investigated.

Effect of chaperones. (i) DnaK/J-GrpE and/or GroEL/ES. The effect of coexpression of DnaK/J-GrpE and/or GroEL/ES is summarized in Table 2 and Fig. 4 using JM109 (pTrcKn29S, pG-KJE8) as the host/vector system. Compared to the control culture with only IPTG supplementation, coexpression of GroEL/ES enhanced proPAC solubility, though with approximately the same low level of specific PAC activity being maintained (i.e., A2 versus A3). While coexpression of DnaK/J-GrpE also enhanced proPAC solubility, PAC activity was significantly increased (i.e., A2 versus A4), suggesting that the post-translational processing for PAC maturation was enhanced. Note that the increased PAC activity was consistent with the increased β-band intensity in the Western blotting result. Simultaneous coexpression of DnaK/J-GrpE and GroEL/ES resulted in a combined effect on enhanced proPAC solubility (i.e., A2 versus A5). However, the specific PAC activity was even decreased compared to the culture with DnaK/J-GrpE coexpression only (i.e., A4 versus A5). Results of both comparisons (i.e., A2 versus A3 and A4 versus A5) suggest that coexpression of GroEL/ES enhanced proPAC solubility but not PAC maturation. By specifically comparing the expression performance (i.e., A4 versus A2+A7), it appears that the improved PAC maturation (i.e., the increased PAC activity)

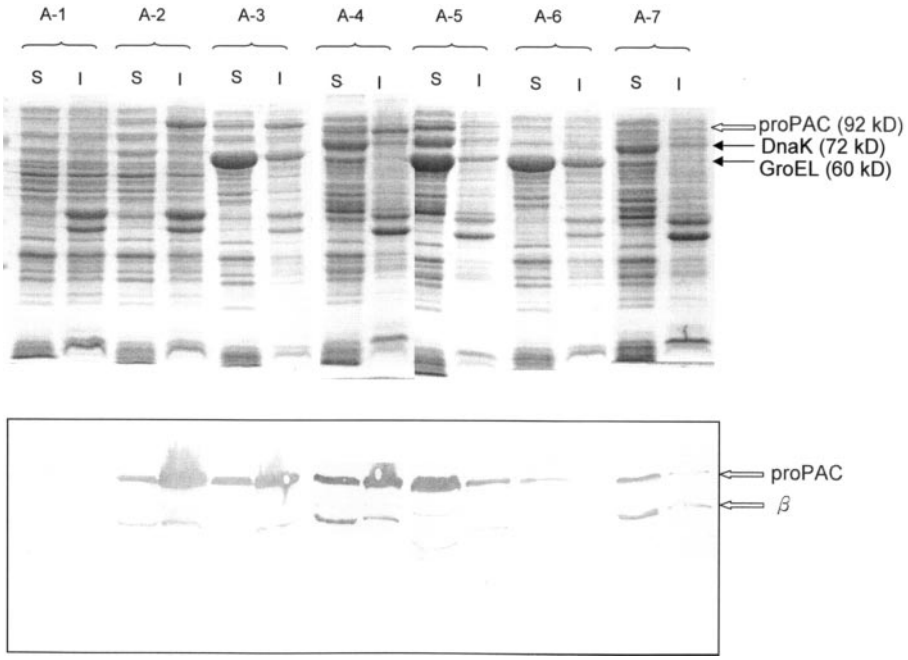

FIG. 4. SDS-PAGE and Western blotting analysis of the soluble (S) and insoluble (I) protein fractions of the culture samples listed in Table 2, i.e., JM109 (pTrcKn29S, pG-KJE8). The gels were made in duplicate for Coomassie blue staining (upper panel) and Western blotting (lower panel). Note that the β subunit, whose band intensity also implied the specific PAC activity, could be identified only in Western blot analysis.

TABLE 3. Expression of LL *pac* for JM109 (pTrcKn29S, pG-Tf3) under various culture conditions[a]

Culture supplement or parameter	Result for culture:						
	B-1	B-2	B-3	B-4	B-5	B-6	B-7
IPTG	−	+	+	+	+	−	−
Tetracycline	−	−	+	−	+	+	−
Arabinose	−	−	−	+	+	−	+
Cell density (OD_{600})	2.3 ± 0.1	2.3 ± 0.1	2.6 ± 0.1	2.9 ± 0.0	3.1 ± 0.2	2.5 ± 0.1	2.9 ± 0.2
Sp act (U/liter/OD_{600})	3 ± 1	35 ± 1	36 ± 2	193 ± 13	156 ± 15	1 ± 1	98 ± 1

[a] Induction of LL *pac*, *groEL/ES*, and *tig* was supposedly effected by the supplementation of IPTG, tetracycline, and arabinose, respectively. +, inducer was added; −, inducer was not used.

for the cultures with DnaK/J-GrpE coexpression through arabinose induction (i.e., A4, A5, and A7) was likely caused by arabinose supplementation rather than DnaK/J-GrpE coexpression. This argument was further confirmed by the expression performance of JM109 (pTrcKn29S) LB-based culture with only arabinose (but not IPTG) supplementation, which had a comparable level of specific PAC activity at 117 U/liter/OD_{600}. In other words, LL *pac* expression under the regulation of the current *trc* promoter system for JM109 (pTrcKn29S) was induced by arabinose and the induction level with respect to PAC activity could be as high as 140-fold, which was much higher than that for IPTG-induced culture (30-fold). Using various arabinose concentrations, it was observed that the induction effect became prominent when the concentration reached a threshold level of 5 g/liter (data not shown). On the contrary, tetracycline itself hardly had any effect on LL *pac* expression (i.e., A1 versus A6).

(ii) Trigger factor and/or GroEL/ES. The effect of coexpression of trigger factor and/or GroEL/ES is summarized in Table 3 and Fig. 5 using JM109 (pTrcKn29S, pG-Tf3) as the host/vector system. Similar to the above results for JM109 (pTrcKn29S, pG-KJE8), coexpression of GroEL/ES enhanced proPAC solubility but not PAC maturation (i.e., B2 versus B3 and B4 versus B5). Coexpression of trigger factor (induced by arabinose) significantly enhanced both proPAC solubility and PAC maturation (i.e., B2 versus B4). It appeared that the proPAC solublization effect mediated by trigger factor was the most prominent among the three chaperones tested in this study. While arabinose also showed the induction effect (i.e., B1 versus B7), a synergistic boost in PAC maturation caused by arabinose supplementation and trigger factor coexpression was observed (i.e., B4 versus B2+B7). The results strongly suggested that trigger factor itself enhances not only proPAC solublization but also PAC maturation in this expression system. This argument was further confirmed using two similar coexpression systems, in which pG-Tf3 was replaced by pTf16 (containing P_{araB}::*tig*) or pG-Tf2 (containing $P_{zt\text{-}lp}$::*groEL/ES*::*tig*) (22; data not shown). Finally, simultaneous coexpression of trigger factor and GroEL/ES resulted in a combined effect on enhanced proPAC solubility (i.e., B5 versus B3+B4)

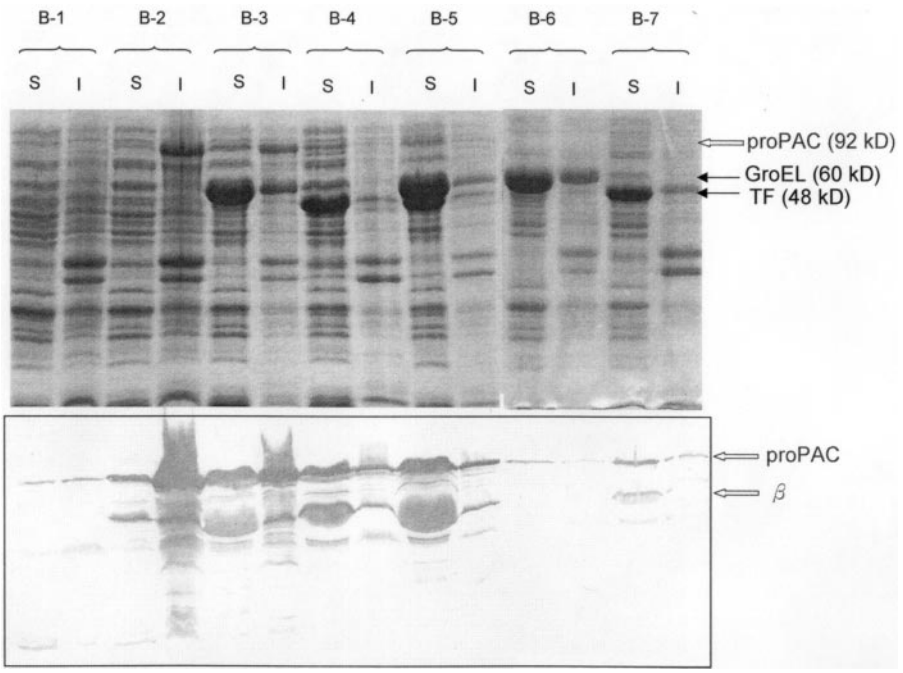

FIG. 5. SDS-PAGE and Western blotting analysis of the soluble (S) and insoluble (I) protein fractions of the culture samples listed in Table 3, i.e., JM109 (pTrcKn29S, pG-Tf3). The gels were made in duplicate for Coomassie blue staining (upper panel) and Western blotting (lower panel). Note that the β subunit, whose band intensity also implied the specific PAC activity, could be identified only in Western blot analysis.

but no extra effect on PAC maturation compared to the culture with trigger factor coexpression only (i.e., B4 versus B5). Again, tetracycline itself hardly had any effect on LL *pac* expression performance (i.e., B1 versus B6).

DISCUSSION

A critical bottleneck limiting the production of PAC is the accumulation of proPAC as inclusion bodies in the periplasm of *E. coli* (8). Apparently, the periplasmic processing machinery was overwhelmed by the excess amount of transiently formed proPAC and the proPAC folding in the periplasm was not properly carried out for subsequent PAC maturation. The search for appropriate intracellular folding modulators that interact in vivo with proPAC provides a valuable means to determine whether the folding/misfolding of proPAC is a critical issue limiting the overproduction of PAC. While it is convincing to find the periplasmic chaperone(s) interacting with proPAC in the periplasm (work currently conducted in this lab), a similar approach could be made to evaluate the effectiveness of various cytoplasmic chaperones given the availability of many expression plasmids containing these cytoplasmic chaperone genes (22). In that case, the processing of proPAC needs to be conducted in the cytoplasm. With the expression of LL *pac* in this study, proPAC could be overproduced and processed to form mature PAC in the cytoplasm, indicating that the posttranslational processing steps for PAC maturation can occur in both the periplasm and the cytoplasm.

Several observations were made on the basis of the LL *pac* expression performance for JM109 (pTrcKn29S) (Fig. 3). First, LL *pac* expression was well regulated by the current *trc* promoter system. Second, LL *pac* overexpression through IPTG induction resulted in the accumulation of proPAC in both soluble and insoluble forms. Third, a portion of induced proPAC could be processed to form active PAC in the cytoplasm, presumably through intramolecular autoproteolysis for PAC maturation (18). Fourth, the formation of insoluble proPAC implies the existence of a protein-folding issue, which could potentially affect not only proPAC solubility but also PAC maturation, for this expression system.

The use of typical cytoplasmic chaperones, including trigger factor, GroEL/ES, and DnaK/J-GrpE, for improving LL *pac* expression was explored. It was shown that all chaperones could assist proPAC folding in vivo and their proPAC solublization effects were summable (Fig. 4 and 5). Trigger factor had the most prominent solublization effect among the three chaperones tested. However, PAC activity was not simultaneously increased with such proPAC solublization assisted by GroEL/ES or DnaK/J-GrpE (Table 2), indicating that the efficiency for PAC maturation was not necessarily improved with proPAC folding. It was proposed that protein synthesis on the ribosome should be coordinated with the activities of various chaperone systems for not only stabilizing nascent polypeptides but also promoting subsequent folding (15). With the peptidyl-prolyl *cis-trans* isomerase activity, trigger factor plays a role in vivo as a ribosome-associated chaperone through the interaction with newly synthesized proteins and a 50S ribosome (13). In this study, coexpression of trigger factor significantly increased PAC activity (Table 3), suggesting that, in addition to its proPAC solublization effect, trigger factor was also able to direct newly synthesized proPAC into the productive pathway for PAC maturation (Fig. 5). It was reported that, on the basis of further association with GroEL, trigger factor can strengthen its function for facilitating protein folding through efficient binding to GroEL substrates (17). In this study, simultaneous coexpression of trigger factor and GroEL/ES had a combined effect on proPAC solublization, but the effect on PAC maturation was minimal compared to the culture with the coexpression of trigger factor only.

It was somewhat surprising to see that the above positive effect on LL *pac* expression partially originated from arabinose supplementation, leading to the argument of arabinose induction for the *trc* promoter system. The induction of the *lac* or *lac*-derived promoter systems (e.g., *tac*, *trc*, etc.) is usually conducted using lactose or its analog, such as IPTG. It was also reported that the *lac* promoter system can be induced by galactose or other galactosides, such as butyl-β-D-galactoside and methyl-β-D-galactoside, which bind to *lac* repressor (5). In this study, we demonstrated that LL *pac* expression under the regulation of the *trc* promoter system could be induced by arabinose (Tables 2 and 3; Fig. 4 and 5), implying that arabinose or one of its derived metabolites could bind to *lac* repressor. The proposed arabinose induction for the *lac* or *lac*-derived promoter systems, which was never reported and would be worthy of investigation, was further confirmed using several other IPTG-inducible gene expression systems in our lab (e.g., P_{trc}::*pac* and DE3/P_{T7}::*pac*) (data not shown). Note that, compared to the cultures with IPTG induction, not only was proPAC overexpressed more preferentially in the soluble form but also the efficiency for PAC maturation was significantly improved upon arabinose induction (e.g., A2 versus A7 or B2 versus B7), resulting in higher PAC activities. Therefore, the current LL *pac* expression system serves as a typical model illustrating that, for the overproduction of proteins in vivo with a series of gene expression steps (i.e., transcription, translation, translocation, and processing), not only should the efficiency of each gene expression step be enhanced but also a balanced flux throughout these protein formation steps should be properly maintained.

On the basis of these results, several further arguments for LL *pac* expression in the cytoplasm could be made. First, the newly synthesized proPAC needed to be soluble for subsequent PAC maturation. The solublization process could be enhanced by the cytoplasmic chaperone of trigger factor GroEL/ES, DnaK/J-GrpE, or their combinations. However, solublization of proPAC did not guarantee PAC maturation. Among the chaperones investigated, trigger factor had the most prominent effect in not only proPAC solublization but also PAC maturation. Since the trigger factor normally interacts with the nascent polypeptide protruding from the ribosome, the enhanced PAC maturation through coexpression of trigger factor suggests that a critical proPAC folding state mediated by trigger factor might be required for maturation. It was proposed that folding in vivo from the ribosome might begin with specific polypeptide conformations, which are dissimilar from early in vitro refolding intermediates and are destined toward the productive folding and assembly pathway (11). In that case, trigger factor might be helpful in terms of preserving this specific folding state for PAC maturation. Furthermore, it was reported that, as a periplasmic protease and

chaperone, DegP could enhance the periplasmic processing for PAC maturation (23). While DegP protease activity was shown to be primarily accountable for the enhancement, we also speculate that part of the DegP function in the periplasm could be similar to that of trigger factor in the cytoplasm, namely, having an interaction with newly synthesized (or newly translocated) proPAC, since DegP is often located on the side of the cytoplasmic membrane facing the periplasm (16) and might be available for immediate interaction with newly translocated proPAC in the periplasm.

The overexpressed proPAC that cannot be immediately processed for maturation tended to aggregate into inclusion bodies and form the major insoluble species. Once trapped in this nonproductive pathway, it appeared difficult for the misfolded proPAC to be reshifted back into the productive pathway for PAC maturation, even though it could be solublized by GroEL/ES or DnaK/J-GrpE, possibly due to the failure in recovering the critical folding state (mediated by trigger factor) of newly synthesized proPAC. The soluble proPAC usually coexisted with mature PAC without another PAC intermediate being detected (Fig. 4 and 5). Such behavior of cytoplasmic maturation was similar to that of periplasmic maturation, for which proPAC was the major insoluble species in the periplasm upon the overexpression of the wild-type *pac* gene (23). The results strongly suggest that the overall PAC maturation was limited by the first autoproteolysis of proPAC and that the formation rate of proPAC from either direct proPAC synthesis out of the ribosome in the cytoplasm (in this study) or translocation of preproPAC into the periplasm (23) could significantly affect the efficiency of this processing step.

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