DegP-Coexpression Minimizes Inclusion-Body Formation upon Overproduction of Recombinant Penicillin Acylase in *Escherichia coli*

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Abstract: We demonstrated the enhancement of recombinant penicillin acylase (PAC) production in *Escherichia coli* by increasing the intracellular concentration of the periplasmic protease DegP. Using appropriate host/vector systems (e.g., HB101 harboring pTrcKnPAC2902 or MDΔP7 harboring pTrcKnPAC2902) in which the expression of the *pac* gene was regulated by the strong *trc* promoter, the overproduction of PAC was often limited by periplasmic processing and inclusion bodies composed of protein aggregates of PAC precursors were formed in the periplasm. The amount of these periplasmic inclusion bodies was significantly reduced and PAC activity was significantly increased upon coexpression of DegP. The specific PAC activity reached an extremely high level of 674 U/L/OD_{600} for MDΔP7 harboring pTrcKnPAC2902 and pKS12 under optimum culture conditions. However, such improvement in the production of PAC was not observed for the expression systems (e.g., MDΔP7 harboring pCLL2902) in which the periplasmic processing was not the step limiting the production of PAC. The results suggest that DegP could in vivo assist the periplasmic processing though the enzyme is shown to be not absolutely required for the formation of active PAC in *E. coli.* In addition, the steps limiting the production of PAC are identified and the reasons for the formation of PAC inclusion bodies are discussed here. *Biotechnol Bioeng* **73**: 484–492, 2001.
Keywords: DegP; *Escherichia coli*; inclusion body; penicillin acylase; periplasm; proteolysis; protease

INTRODUCTION

Through successful applications of recombinant DNA technology, it is now feasible to produce desired proteins in large amounts using appropriate gene-expression systems. *Escherichia coli* is still the most common host for recombinant protein production. The well-known genetic information of *E. coli* makes it possible for a variety of attempts using genetic engineering, metabolic engineering, and protein-engineering techniques. On the other hand, the fast growth rate and the ease of cultivation technology for *E. coli* make it suitable for industrial application. Upon performing the cultivation for recombinant protein production using various strategies (Makrides, 1996), there are at least two goals to be targeted. The first one is high-cell-density cultivation, while the second is high-level gene expression. Culture performance for recombinant protein production can be optimized when the two goals are simultaneously attained. High-cell-density culture can be obtained using fed-batch cultivation (Yee and Blanch, 1992), in which the concentrated medium is gradually fed into the bioreactor. The primary concern of this operation is to develop an optimum feeding strategy, based on which cells can be maintained at a high-energy state for enhancing recombinant protein production while the culture cell density is increasing. Various genetic strategies have been developed toward high-level gene expression. The use of a strong promoter (Sawers and Jarsch, 1996), such as *tac*, for regulation of the gene expression has been successfully applied to improve the transcriptional efficiency (perhaps the translational efficiency as well) and enhance recombinant protein production. However, it is common that insoluble protein aggregates, known as inclusion bodies, tend to accumulate in cells upon the overproduction of gene products (Kane and Hartley, 1988; Strandberg and Enfors, 1991). The potential of this application becomes limited because renaturation of these misfolded proteins is usually difficult (Buchner et al., 1992; Hsih et al., 1997).

The mechanism of the inclusion-body formation is not completely understood. There are reasons to believe that the overexpressed gene products cannot be suitably processed by folding modulators (Thomas et al., 1997) for developing the proper protein structure. For proteins destined to be exported, the protein-formation mechanism would be more complicated and the efficiency of translocation, post-translocational folding and processing becomes important. It is possible for both cytoplasmic and periplasmic precursors (i.e., protein intermediates) to form inclusion bodies upon protein overproduction (Bowden et al., 1991). This raises an important issue that, for the overproduction of

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recombinant proteins, a "balanced" protein synthesis flux throughout the gene expression steps (i.e., transcription, translation, and post-translational steps) should be properly maintained to avoid the accumulation of polypeptide intermediates in addition to improving the efficiency of each step.

We have developed various strategies for the heterologous overproduction of penicillin acylase (PAC), which is an important industrial enzyme for the production of many β-lactam antibiotics (Shewale et al., 1990; Shewale and Sivaraman, 1989), in *E. coli.* The mature PAC is located in the periplasm and the formation of PAC requires a series of post-translational steps (Sizmann et al., 1990), including translocation and periplasmic processing/folding steps, which are unusual for prokaryotic proteins (Fig. 1). Recombinant DNA technology has been applied for overproducing PAC in *E. coli* (Hunt et al., 1990; Scherrer et al., 1994; Sobotkova et al., 1995; Sobotkova et al., 1996; Sriubolmas et al., 1997). However, it is a challenging task to systematically develop expression strategies for improving the production of PAC primarily due to incomplete genetic information on the mechanisms involved in protein formation (Kasche et al., 1999; Sizmann et al., 1990) and/or regulation (Keilmann et al., 1993; Merino et al., 1992; Roa and Garcia, 1999) with respect to the *pac* gene expression. Our strategies for the overproduction of PAC mainly focused on optimizing the host/vector system. For the expression vector, we constructed several plasmids with improved transcriptional and translational efficiency for the *pac* gene expression (Chou et al., 1999d). With these plasmids, we also demonstrated that the production of recombinant PAC could be limited by various gene-expression steps, including transcription, translation, and post-translational steps (Chou et al., 1999d). For the expression host, we developed a novel strategy for the screening of *E. coli* mutants with improved

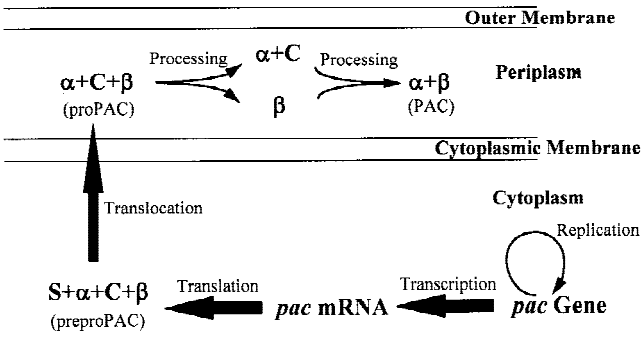

Figure 1. Synthesis and maturation of PAC in *E. coli.* The structural *pac* gene from *E. coli* ATCC11105 encodes a polypeptide precursor (preproPAC) which has a molecular weight of approximately 95 kD and is composed of, in the direction of N-terminus to C-terminus, a signal peptide (S), α subunit (α), connecting peptide (C), and β subunit (β). The signal peptide directs the export of preproPAC into the periplasm and is removed to form another type of PAC precursor (proPAC at 92 kD) after translocation. Periplasmic processing follows to remove the connecting peptide, and the two subunits (α at 24 kD and β at 62 kD) are available for assembling mature PAC. The periplasmic processing is initiated by cleavage of the Thr_{263}-Ser_{264} bond linking C and β (mentioned in Results and Discussion).

ability for the *pac* gene expression (Chou et al., 1999c). The use of the improved host/vector systems resulted in a significant enhancement on the production of PAC.

The formation of inclusion bodies, which are primarily composed of PAC precursors in the periplasm, was recently identified as an important obstacle in the overproduction of PAC in *E. coli* (Scherrer et al., 1994; Sriubolmas et al., 1997). The same process bottleneck occurred in our host/vector systems, such as MDΔP7 harboring pTrcKnPAC2902, in which the strong *trc* promoter system was used for regulating the *pac* gene expression. Hence, efforts have been directed toward reducing the amount of periplasmic inclusion bodies. In the present study, the effect of the expression of a periplasmic protease DegP on the overproduction of PAC and formation of periplasmic inclusion bodies in *E. coli* was investigated. The approach was founded on the basis of the hypothesis that the periplasmic processing machinery was saturated by the overproduced PAC precursors and the production of PAC was therefore limited by the processing. The periplasmic processing mechanism has been known to consist of various proteolytic steps though it is not fully characterized. The proteolyses are expected to be carried out either by periplasmic protease or via intramolecular autoproteolysis (Kasche et al., 1999; Sizmann et al., 1990). DegP is a characterized periplamsic protease and is required for proteolysis in the cell envelope of *E. coli* (Strauch et al., 1989). Because DegP is located in the periplasm, there is a chance that the enzyme could possibly be involved in the periplasmic processing of PAC precursors. We demonstrated that, by increasing the intracellular DegP concentration, the majority of the overproduced PAC precursors could be smoothly processed for maturation and the amount of periplasmic inclusion bodies (i.e., protein aggregates of proPAC) was significantly reduced. The results suggest that the critical step limiting the production of PAC (i.e., the proteolysis on the Thr_{263}-Ser_{264} bond of proPAC) became more efficient upon the *degP* expression. Most importantly, PAC activity was significantly increased and an extremely high *pac* gene-expression level of 674 U/L/OD_{600} was reached for MDΔP7 harboring pTrcKnPAC2902 and pKS12 under optimum culture conditions. Based on the results, the step limiting the production of PAC was clearly identified and the strategy for resolving the problem of inclusion body formation was effectively proposed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Strains and plasmids used in the study are summarized in Table I and briefly described here. HB101 (Boyer and Roulland-Dussoix, 1969) and its derived mutant MDΔP7 (Chou et al., 1999c) were used as the host for the production of recombinant PAC in a bioreactor. Compared to HB101, MDΔP7 could potentially have a higher *pac* translational

Table I. Summary of *E. coli* strains and plasmids used in this study.

Strain and plasmid	Relevant genotype or phenotype	Source and reference
E. coli		
HB101	F⁻ *hsdS20 leuB6 recA13 ara-14 proA2 lacY1 thi-1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ⁻	CCRC[a] (Boyer and Roulland-Dussoix, 1969)
MDΔP7	A penicillin-sensitive and 6-APA-resistant mutant (derived from HB101) with a high PAC-producing capability	This lab (Chou et al., 1999c)
KS272	F⁻ Δ*lacX74 galE galK thi rpsL (strA)* Δ*phoA (PvuII)*	J. Beckwith (Strauch et al., 1989)
KS474	KS272 *degP41*	J. Beckwith (Strauch et al., 1989)
S. marcenscens		
ATCC27117	A penicillin-G-resistant and 6-APA-sensitive strain	ATCC
Plasmid		
pCLL2902	An expression vector containing the 2.9-kb *pac* operon from ATCC11105, Ori (pBR322), Tc®	This lab (Chou et al., 1999d)
pCLL3201	An expression vector containing the 3.2 kb *pac* operon from ATCC11105, Ori (pBR322), Tc®	This lab (Chou et al., 1999d)
pKS12	An expression vector containing the *degP* gene, Ori (pACYC184), Cm®	J. Beckwith (Strauch et al., 1989)
pTrcKnPAC2902	An expression vector containing the *pac* operon (from pCLL2902) fused with the *trc* promoter, Ori (pBR322), Km®	This lab (Chou et al., 1999d)
pTrcKnPAC3221p	An expression vector containing the *pac* operon (without the native *pac* promoter and with a modified RBS) fused with the *trc* promoter, Ori (pBR322), Km®	This lab (Chou et al., 1999d)

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

and periplasmic processing efficiency for the *pac* gene expression (Chou et al., 1999c). KS272 and KS474 were used for investigating the *degP* mutation effect on the production of PAC. The plasmid of pKS12 contains the *E. coli degP* gene (Strauch et al., 1989). The plasmids of pCLL2902 and pCLL3201 contain the *pac* operon whose transcription is regulated by the native *pac* promoter (Chou et al., 1999d). On the other hand, the plasmids of pTrcKnPAC2902 and pTrcKnPAC3221p contain the *pac* operon whose transcription is regulated by the *trc* promoter (Chou et al., 1999d). All the above *pac* gene expression vectors have the pBR322 replication origin, therefore, are compatible with the *degP* gene expression vectors pKS12 which has the pACYC184 replication origin. Purification of plasmid DNAs was performed using a spin-column kit (Clontech, Palo Alto, CA). Plasmid transformation was carried out according to Chung and Miller (1993) or using an electroporator (*E. coli* Pulser, Bio-Rad, Hercules, CA).

Batch Cultivation in a Shaker

Cells were revived by streaking of the stock culture stored at −80°C on an LB agar plate (5 g/L NaCl, 5 g/L Bacto yeast extract, 10 g/L Bacto tryptone, and 15 g/L Bacto agar). The plate was incubated at 37°C for approximately 20 h. An isolated single colony was picked to inoculate 25 mL of MPAC medium (5 g/L NaCl, 5 g/L Bacto beef extract, and 10 g/L Bacto yeast extract), which was then incubated at 37°C on a rotary shaker at 220 rpm for approximately 15 h. The medium was supplemented with tetracycline (Tc) at 10 μg/mL, kanamycine (Kn) at 25 μg/mL or chloramphenicol (Cm) at 32 μg/mL when necessary. Four milliliters of the seed culture was used to inoculate 250-mL Erlenmeyer flasks containing 50 mL MPAC medium. Batch cultivation was conducted in a reciprocating shaker at 200 rpm and 28°C for approximately 36 h.

Batch Cultivation in a Bioreactor

Cells were revived and the seed culture was prepared as described above. The seed culture at a volume of 10 mL was used to inoculate a lab-top bioreactor (VirTis, Gardiner, NY; Omni-Culture) containing 1-L working volume of MPAC medium. Unless specified, isopropyl β-D-thiogalactopyranoside (IPTG) at 0.05 m*M* was added to induce the synthesis of PAC. The culture was supplemented with 10 μL/L antifoam 289 (Sigma, St. Louis, MO) to avoid excessive foaming. Filter-sterilized air at 1.5 L/min was purged into the culture for aeration. The culture pH was regulated at 7.0 ± 0.1 by adding 3 N NaOH or 3 N HCl using a combined pH electrode (Mettler-Toledo, Switzerland), a pH controller (Suntex, Taipei, Taiwan, Republic of China; PC310), and two peristaltic pumps (Watson Marlow, Falmouth, UK; 101U/R). The bioreactor was operated at 28°C and 500 rpm for approximately 48 h.

Treatment of Culture Sample

The culture sample was appropriately diluted with saline solution for measuring cell density using a spectrophotometer (Jasco, Tokyo, Japan, Model V-530) set at 600 nm (OD_{600}). For the preparation of cell extract, the culture sample of 10 mL was centrifuged at 2°C and 6000*g* for 5 min. The supernatant was assayed for the extracellular PAC activity. The cell pellet was resuspended in 2 mL of sodium phosphate buffer (0.05*M*, pH 7.5). The cell suspension was sonicated for 2 min using an ultrasonic processor (Sonics & Materials, Danbury, CT) and then centrifuged at 2°C and

20,000*g* for 20 min. The supernatant containing soluble proteins was assayed for the intracellular PAC activity. The pellet containing insoluble proteins and cell debris was 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. The protein content of the pellet (as the insoluble fraction) was analyzed by SDS-PAGE and Western blotting.

Microbiological Assay for 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 resistent to penicillin but sensitive to 6-APA (Meevootisom et al., 1983).

PAC Enzyme Assay

PAC 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). All assays were conducted in duplicate. One unit was defined as the amount of enzyme that hydrolyzed 1.0 μmole penicillin G per min at 37°C. The volumetric activity (in U/L) is the product of the specific activity (in U/L/OD_{600}) and cell density (in OD_{600}).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunological Analysis (Western Blotting)

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) according to the manufacturer's instructions. Protein samples at 0.4 OD_{600} unit for the insoluble fractions of the cell extracts were loaded for analysis. The electrophoresis was conducted under a constant voltage of 200 volts for at least 30 min. After SDS-PAGE, proteins on the polyacrylamide gel were electroblotted to a PVDF membrane using a transfer electrophoresis unit (Hoefer, San Francisco, CA; TE 22) according to a standard protocol (Towbin et al., 1979). The electrophoretic transfer was conducted at a constant current of 0.4 A 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 rabbit and 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 horseradish peroxidase (HRP). PAC-related polypeptides were detected by a colorimetric method using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the substrate. The processed membranes were scanned.

RESULTS AND DISCUSSION

Effect of DegP Coexpression on Production of PAC for Various Host/Vector Systems

Using HB101 harboring pTrcKnPAC2902, in which the expression of the *pac* gene was regulated by the *trc* promoter system (Chou et al., 1999d), the bioreactor performance on the production of PAC is summarized in Figure 2A. Formation of PAC inclusion bodies was observed (Fig. 2C). The inclusion bodies were localized in the periplasm according to previous reports (Chou et al., 1999b; Scherrer et

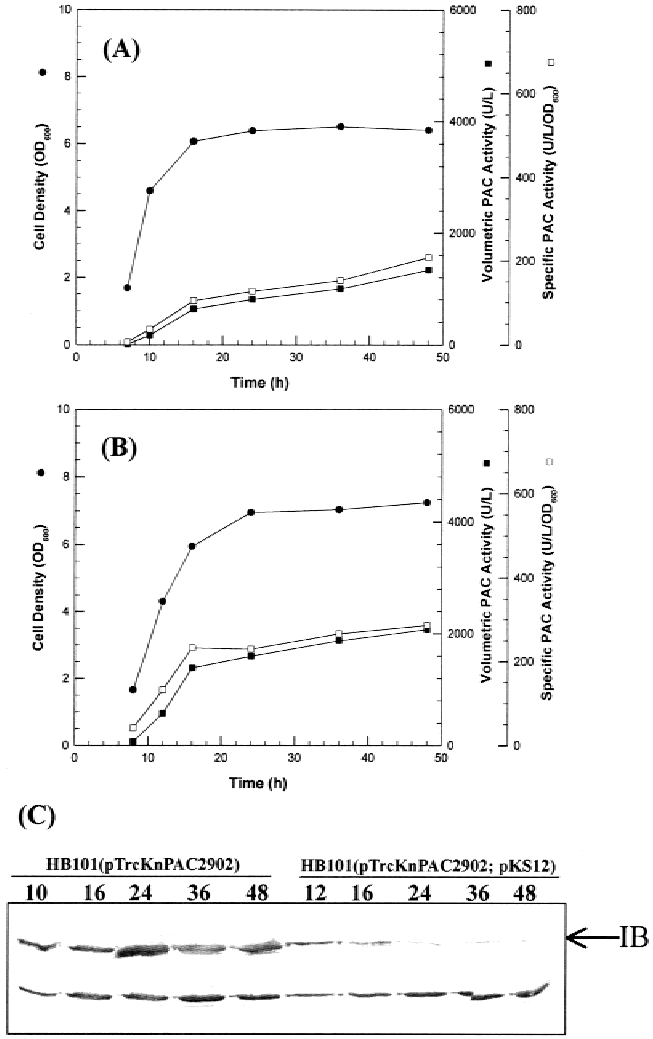

Figure 2. Production of PAC for HB101 harboring pTrcKnPAC2902 (Panel A) and HB101 harboring pTrcKnPAC2902 and pKS12 (Panel B). Time profiles for cell density, specific PAC activity, and volumetric PAC activity are shown. Formation of PAC inclusion bodies for various samples is shown in Panel C. The number of each lane represents the timing at which the sample was taken. For Figures 2–4, IPTG at 0.05 m*M* was added when the first sample was taken (i.e., OD_{600} of 1.5–2.0).

al., 1994; Sriubolmas et al., 1997). Coexpression of DegP using HB101 harboring pTrcKnPAC2902 and pKS12 resulted in a significant decrease in the amount of inclusion bodies (Fig. 2C), suggesting that the periplasmic processing machinery was no longer saturated. The final volumetric and specific PAC activities of the binary-plasmid HB101 were increased by 50% and 40%, respectively (Fig. 2B), compared to the single-plasmid HB101. The results indicate that the over-accumulated precursors (i.e., proPAC) were appropriately processed for maturation when DegP was co-expressed.

MDΔP7 is a mutant derived from HB101 using a PAC-specific host screening method (Chou et al., 1999c). The bioreactor performance on the production of PAC for MDΔP7 harboring pTrcKnPAC2902 and MDΔP7 harboring pTrcKnPAC2902 and pKS12 is summarized in Figure 3. Compared to HB101 harboring pTrcKnPAC2902, the final volumetric and specific PAC activities of MDΔP7 harboring pTrcKnPAC2902 were increased by 140% and 90%, respectively (Figs. 2A and 3A). However, the amount of inclusion bodies was also significantly increased (Figs. 2C and 3C). The results suggest that the efficiency of the *pac* translation as well as post-translational steps for the mutant strain MDΔP7 was significantly higher than that of the parent strain HB101. However, the production of PAC for MDΔP7 harboring pTrcKnPAC2902 was still limited by periplasmic processing steps. Similar to the above results using HB101 as the host, not only the amount of inclusion bodies was significantly reduced (Fig. 3C) but also PAC activity was significantly increased for MDΔP7 harboring pTrcKnPAC2902 and pKS12 (Fig. 3B), compared to MDΔP7 harboring pTrcKnPAC2902. The results again indicate that DegP could enhance the processing of the over-produced PAC precursors in the periplasm, and this function of enhancement appeared to be independent of expression host.

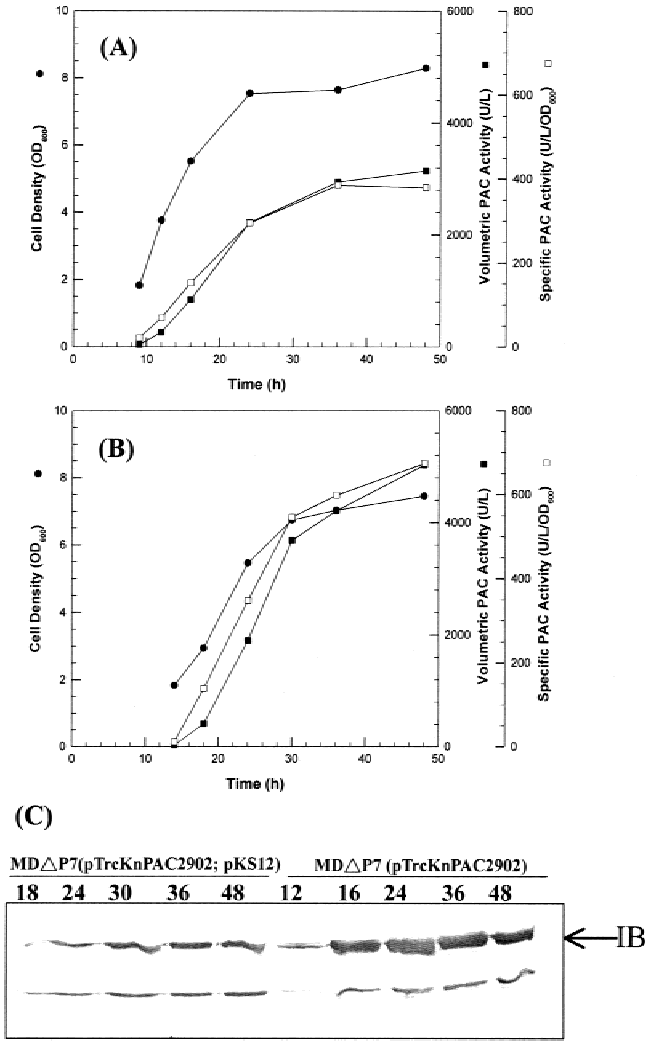

Figure 3. Production of PAC for MDΔP7 harboring pTrcKnPAC2902 (Panel A) and MDΔP7 harboring pTrcKnPAC2902 and pKS12 (Panel B). Formation of PAC inclusion bodies for various samples is shown in Panel C. Details are as outlined in the Figure 2 legend.

Two other plasmids, pTrcKnPAC3221p and pCLL2902, were also used for investigating the effect of DegP on the production of PAC. The plasmid structure of pTrcKnPAC3221p was similar to that of pTrcKnPAC2902 except that the native *pac* promoter in pTrcKnPAC3221p was removed and the *pac* gene expression was completely regulated by the *trc* promoter (Chou et al., 1999d). Formation of inclusion bodies at a large amount was observed for MDΔP7 harboring pTrcKnPAC3221p (Fig. 4C). The problem was resolved by the *degP* expression using MDΔP7 harboring pTrcKnPAC3221p and pKS12. Similar to the above results, both the reduction in the amount of inclusion bodies and the increase in PAC activity for MDΔP7 harboring pTrcKnPAC3221p and pKS12 were significant, compared to MDΔP7 harboring pTrcKnPAC3221p (Fig. 4). However, the DegP function on improving the periplamsic processing was not observed when pCLL2902 was used as the *pac* expression plasmid. The expression of the *pac* gene was regulated by the native *pac* promoter, which is rather weak compared to the *trc* promoter, for pCLL2902 (Chou et al., 1999d). The bioreactor performances on the production of PAC for MDΔP7 harboring pCLL2902 and MDΔP7 harboring pCLL2902 and pKS12 were approximately the same (Fig. 5). The results were not surprising because the formation of inclusion bodies was not observed for the two expression systems (data not shown), indicating that the periplasmic processing no longer limited the production of PAC. Similar results were obtained for the experiments using MDΔP7 harboring pTrcKnPAC2902 induced with a lower concentration of IPTG (e.g., 0.02 m*M*). The improvement in culture performance due to coexpression of *degP* was minor because PAC inclusion bodies at a minimum amount were formed (data not shown). The results suggest that the DegP function on the *pac* expression was dependent on the *pac* expression performance, which is related to expression systems or culture conditions. The improvement on the production of PAC could be observed only for the expression systems in which the production of PAC was limited by periplasmic processing.

Approximately 5–10% of total PAC activity was de-

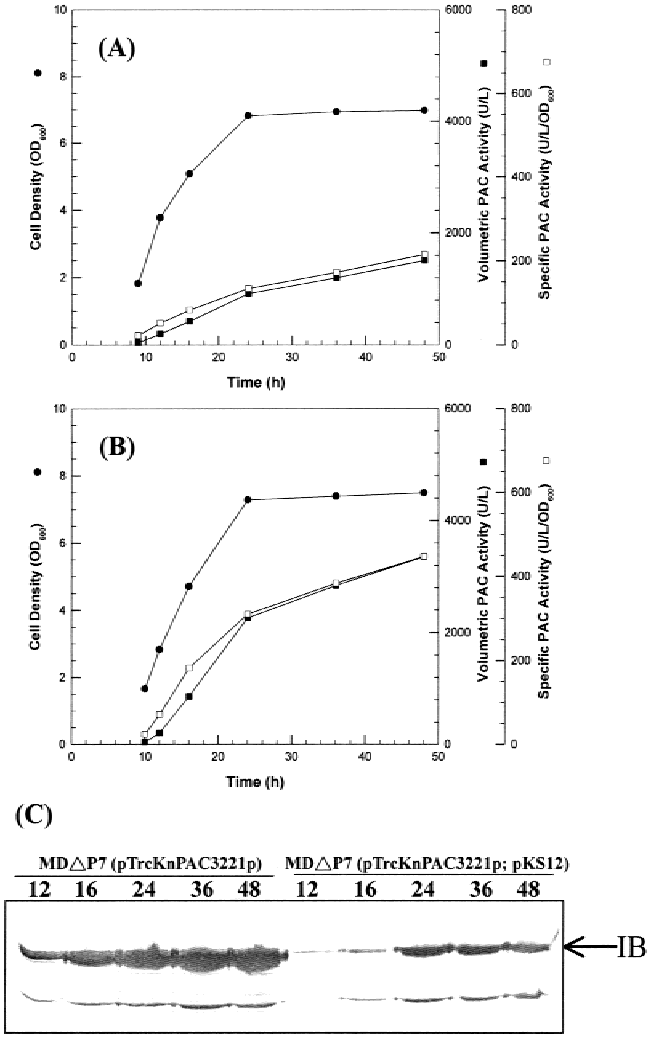

Figure 4. Production of PAC for MDΔP7 harboring pTrcKnPAC3221p (Panel A) and MDΔP7 harboring pTrcKnPAC3221p and pKS12 (Panel B). Formation of PAC inclusion bodies for various samples is shown in Panel C. Details are as outlined in the Figure 2 legend.

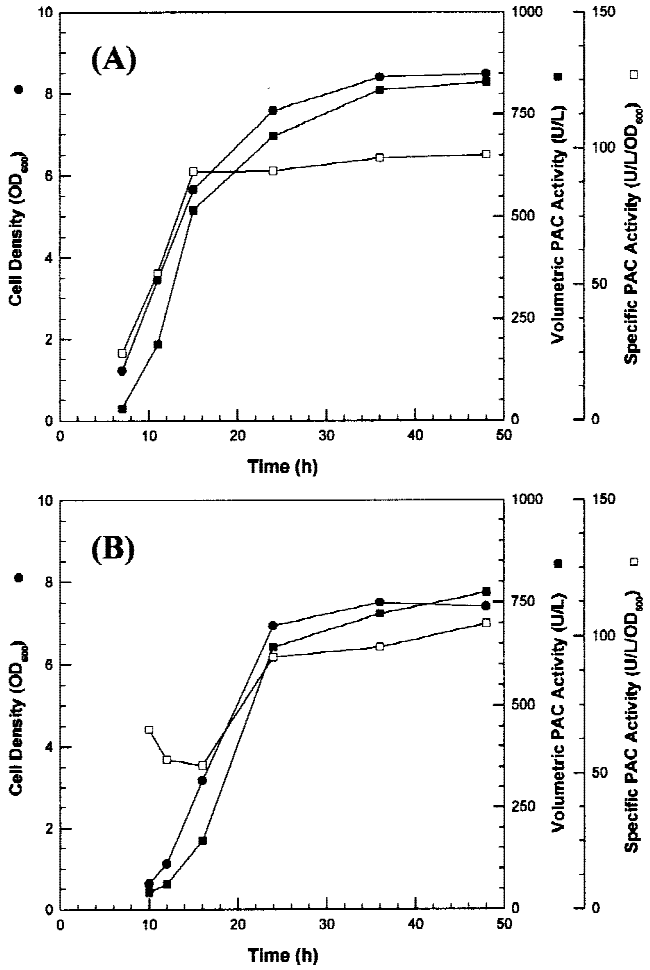

Figure 5. Production of PAC for MDΔP7 harboring pCLL2902 (Panel A) and MDΔP7 harboring pCLL2902 and pKS12 (Panel B). Details are as outlined in the Figure 2 legend. No IPTG was added.

tected in the extracellular fraction for strains harboring pTrcKnPAC-plasmid (e.g., the cases of Figs. 2A, 3A, and 4A, data not shown), indicating that some cells could lyse upon the overexpression of the *pac* gene. On the other hand, minimum PAC activity was detected in the extracellular fraction for strains harboring pTrcKnPAC- and pKS12 (e.g., the cases of Figs. 2B, 3B, and 4B, data not shown). The results suggest that the toxicity from the overproduced *pac* gene products was minimized and cell physiology was improved by the expression of *degP*. The improvement in cell physiology can be clearly observed in Figure 6. In these experiments, the expression of *pac* was highly induced with IPTG of 0.2 m*M*. For MDΔP7 harboring pTrcKnPAC2902, cell growth was severely inhibited due to toxicity from the fast-accumulated *pac* gene products (Fig. 6A). Inclusion bodies at an extremely large amount were formed (Fig. 6C). Although the total specific PAC activity (i.e., the sum of intracellular and extracellular PAC activities) was relatively high (~600 U/L/OD_{600}), approximately 50% of total PAC activity was detected in the extracellular fraction; indicating a significant amount of cells lysed. These problems were resolved by the *degP* expression using MDΔP7 harboring pTrcKnPAC2902 and pKS12 under the same culture conditions. Cell growth was not inhibited, formation of inclusion bodies was minimized, and the production of PAC was significantly enhanced possibly due to improved cell physiology (Fig. 6B).

Possible Roles of DegP on Production of PAC

The above results strongly suggest that DegP could be involved in the periplasmic processing of PAC precursors. We were interested in seeing whether DegP is a specific enzyme in the periplasmic-processing pathway. To do this, the expression of *pac* for a selection of expression systems was carried out using the wild-type strain (KS272) or *degP* mutant (KS474) as the host, pCLL2902 or pCLL3201 as the *pac* expression vector, and pKS12 as the *degP* expression vector for complementing the *degP* mutation of the expression host. The results of shake-flask experiments are sum-

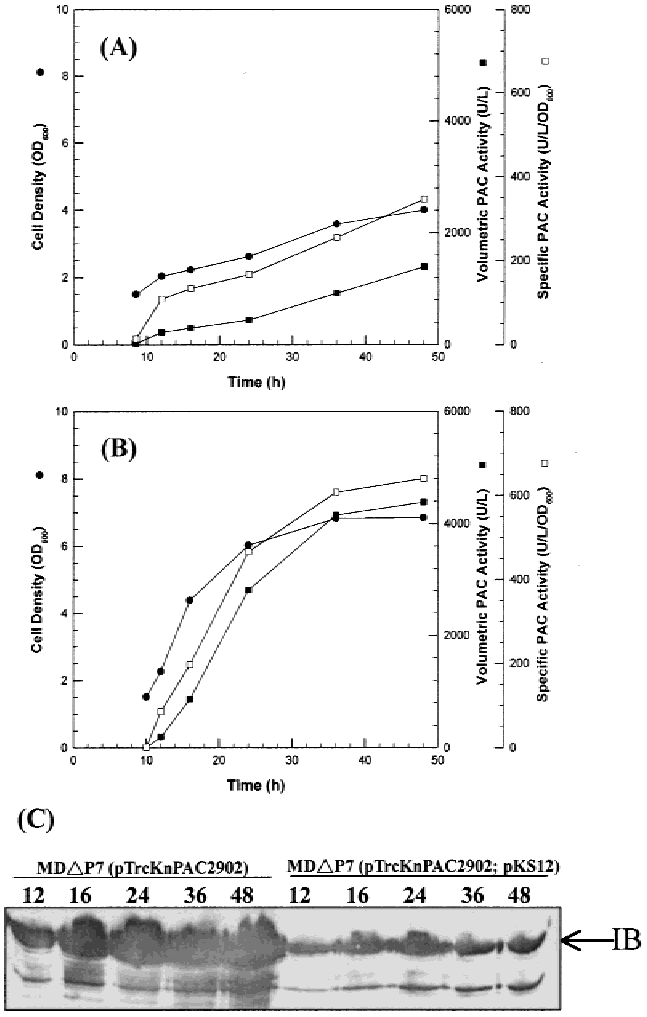

Figure 6. Production of PAC for MDΔP7 harboring pTrcKnPAC2902 (Panel A) and MDΔP7 harboring pTrcKnPAC2902 and pKS12 (Panel B). Formation of PAC inclusion bodies for various samples is shown in Panel C. Details are as outlined in the Figure 2 legend. IPTG at 0.2 m*M* was added when the first sample was taken.

marized in Table II. By qualitative microbiological test (data not shown) or quantitative PAC assay (Table II), there was no significant difference in PAC activity among the strains of KS272 harboring pCLL3201, KS474 harboring pCLL3201, and KS272 harboring pCLL3201 and pKS12; indicating that DegP was not absolutely required for the formation of active PAC in *E. coli.* However, the *pac* gene expression vector pCLL2902 could not be stably maintained in the *degP* mutant KS474 for unknown reasons. This defect in plasmid propagation could be related to the *degP* mutation in KS474 because it was complemented by the plasmid of pKS12, which contains the wild-type *degP* gene. In other words, pCLL2902 could be stably maintained in KS474 when *degP* was expressed (from pKS12). PAC activity for KS272 harboring pCLL2902 was approximately the same as that for KS474 harboring pCLL2902 and pKS12 (Table II). The results indirectly suggest that there might be multiple pathways for processing PAC precursors in the periplasm and DegP could possibly assist, rather than specifically catalyze, the periplasmic processing.

It was reported that the production of PAC was limited by periplasmic-processing steps when the *pac* expression was regulated by a strong promoter, and the overproduced PAC precursors (i.e., proPAC) tended to accumulate in the periplasm as inclusion bodies (Chou et al., 1999b; Scherrer et al., 1994; Sriubolmas et al., 1997). At least two reasons could account for the observations. First, the periplasmic folding of PAC precursors was not properly carried out, possibly due to an increased level of the local protein concentration upon the *pac* overexpression. Second, the periplasmic-processing machinery could be saturated by the overproduced PAC precursors and, therefore, limited the production of PAC. We believe that only the second viewpoint was supported by the current study and our recent experiments, in which bacteriocin release protein (BRP), a protein that has been successfully applied to extracellularly release periplasmic proteins (van der Wal et al., 1995a; van der Wal et al., 1995b), was coexpressed with PAC in *E. coli.* The amount of PAC inclusion bodies was not reduced though active PAC was released into the medium via BRP-mediated secretion. The results suggest that the formation of inclusion bodies was not caused by overaccumulation of acitve PAC that blocked periplasmic processing (manuscript submitted).

The periplasmic processing of proPAC is initiated by the proteolysis on the Thr_{263}-Ser_{264} bond. The two protein chains (i.e., α + C and β in Fig. 1) are formed and fold with each other. Enzyme activity at approximately the same level as that of mature PAC could be detected for the folding complex (Lindsay and Pain, 1990;1991). The connecting peptide was chopped by subsequent proteolyses. However, the protease(s) and mechanism for periplasmic processing have not been identified. Sizmann et al. (1990) argued that the processing is probably not carried out by a specific

Table II. Effect of the *degP* mutation on the production of PAC.

Host Plasmid	KS272 pCLL2902	KS474 pCLL2902	KS474 pCLL2902 pKS12	KS272 pCLL3201	KS474 pCLL3201	KS474 pCLL3201 pKS12
Cell density (OD_{600})[a]	5.4 ± 0.0	NA[b]	5.6 ± 0.4	5.6 ± 0.3	5.6 ± 0.4	5.2 ± 0.1
Specific PAC activity (U/L/OD_{600})[a]	77.4 ± 3.6	NA[b]	94.3 ± 6.1	39.6 ± 7.3	25.7 ± 2.1	35.9 ± 1.0

[a]Results are averages of three identical experiments.
[b]NA, not available due to failure of plasmid propagation.

processing enzyme(s) because active *E. coli* PAC could be expressed in other bacterial systems. The argument seems to be consistent with the current results. Based on the above arguments and available results (Chou et al. 1999b; Scherrer et al., 1994; Sriubolmas et al., 1997), the production of PAC was likely limited by the proteolysis on the Thr_{263}-Ser_{264} bond, which was conducted either by periplasmic protease or via intramolecular autoproteolysis (Kasche et al., 1999). The current results indirectly indicate that the proteolysis on the Thr_{263}-Ser_{264} bond could be enhanced by increaisng the intracellular DegP concentration, though DegP was not absolutely required for periplasmic processing. The amount of periplasmic protein aggregates was significantly reduced, indicating that the overproduced precursors (i.e., proPAC) were smoothly processed for maturation. The results suggest that the DegP concentration in the current expression systems was high enough for processing the over-accumulated periplasmic PAC precursors and the successful DegP-mediated processing resulted in the enhanced production of active PAC. Finally, note that DegP, as a periplasmic protease for assisting the proteolysis of proPAC, would not cause degradation of mature PAC and affect the stability of PAC (Panel B in Figs. 2–6). This is important for the current strategy to be applied in the overproduction of active recombinant PAC.

CONCLUSIONS

Upon the overproduction of recombinant proteins, the cell physiology tends to be seriously affected and the foreign gene products are subjected to intracellular proteolyses. Therefore, various genetic strategies based on minimizing intracelular proteolysis were developed (e.g., using protease-deficient strains) for improving recombinant protein production (Enfors, 1992; Gottesman, 1990; Meerman and Georgoiu, 1994). Contradictory to this common strategy, the present study demonstrated the enhancement of recombinant protein production by increasing the intracellular (i.e., periplasmic) protease concentration. This was because that the target protein of PAC is an exported protein and requires a series of proteolyses for maturation. The overproduction of PAC was often limited by periplasmic processing and the over-accumulated PAC precursors formed inclusion bodies in the periplasm. The periplasmic protease DegP could assist the periplasmic processing though the enzyme was not absolutely required for the formation of active PAC. Cell physiology was significantly improved by coexpression of *degP* in the PAC-overproducing strains. It appeared that only proPAC but not mature PAC would be the proteolytic target for DegP. Hence, the stability of recombinant PAC was not affected by the expression of *degP*. By increasing the intracellular DegP concentration, the amount of periplasmic inclusion bodies was significantly reduced and the production of PAC was no longer limited by periplasmic processing. The effect of DegP on the expression of the *pac* gene was dependent on the expression system. In other words, the improvement on the production of PAC could be observed only for the expression systems in which the production of PAC was limited by periplasmic processing. Both volumetric PAC activity (at 5025 U/L) and specific PAC activity (at 674 U/L/OD_{600}) for MDΔP7 harboring pTrcKnPAC2902 and pKS12 were significantly increased, compared to MDΔP7 harboring pTrcKnPAC2902, under optimum culture conditions. Note that this extremely high PAC activity was approximately 600-fold that of the noninduced culture of the wild-type PAC producer ATCC111105 (Chou et al., 1999a), and the expresion system could be of great interest for industrial application. In addition to the increase in PAC activity, we also demonstrated an important concept of developing genetic strategy for improving recombinant protein production; that is, the bottleneck step(s) limiting recombinant protein production needs to be precisely identified. To our knowledge, the strategy of using protease for reducing the amount of inclusion bodies and improving recombinant protein production has never been exploited and this study is probably the first approach. The use of PAC, which has such an unusual complex protein formation mechanism, as the model protein made this demonstration possible.

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