ARTICLE

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Effect of Heat-Shock Proteins for Relieving Physiological Stress and Enhancing the Production of Penicillin Acylase in *Escherichia coli*

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ABSTRACT: High-level expression of recombinant penicillin acylase (PAC) using the strong *trc* promoter system in *Escherichia coli* is frequently limited by the processing and folding of PAC precursors (proPAC) in the periplasm, resulting in physiological stress and inclusion body formation in this compartment. Periplasmic heat-shock proteins with protease or chaperone activity potentially offer a promise for overcoming this technical hurdle. In this study, the effect of the two genes encoding periplasmic heat-shock proteins, that is *degP* and *fkpA*, on *pac* overexpression was investigated and manipulation of the two genes to enhance the production of recombinant PAC was demonstrated. Both Δ*degP* and Δ*fkpA* mutants showed defective culture performance primarily due to growth arrest. However, *pac* expression level was not seriously affected by the mutations, indicating that the two proteins were not directly involved in the pathway for periplasmic processing of proPAC. The growth defect caused by the two mutations (i.e., Δ*degP* and Δ*fkpA*) was complemented by either one of the wild-type proteins, implying that the function of the two proteins could partially overlap in cells overexpressing *pac*. The possible role that the two heat-shock proteins played for suppression of physiological stress caused by *pac* overexpression is discussed.

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KEYWORDS: recombinant protein production; gene expression; penicillin acylase; heat-shock protein; chaperone; cell physiology

Introduction

Various biochemical and genetic strategies have been developed toward high-level recombinant protein production in *Escherichia coli* (Andersen and Krummen, 2002; Baneyx, 1999; Makrides, 1996). While heterologous proteins are first synthesized in the cytoplasm, they can be subsequently targeted to various extracytoplasmic compartments (Cornelis, 2000), such as the periplasm (Jobling et al., 1997), cell surface/outer membrane (Lee et al., 2003), and even extracellular medium (Georgiou and Segatori, 2005). Periplasm has an oxidizing environment and relevant enzymes for the formation of disulfide bonds that are required for many eukaryotic proteins. Upon the cleavage of signal peptides, proteins with authentic N-terminal sequences can be expressed in the periplasm of *E. coli*. Since the number of proteins in the periplasm is significantly less than that in the cytoplasm where most intracellular proteins reside, targeting the expressed foreign gene products in the periplasm will facilitate subsequent protein purification. Recently, technical advances related to gene expression in the periplasm have been highly emphasized. For example, effort has been made to design leader sequences for effective translocation of protein precursors across the inner membrane (Jobling et al., 1997). Similar to what occurs in the cytoplasm, protein folding has been recognized as a common problem (Baneyx and Mujacic, 2004) for the overproduction of gene products in the periplasm since periplasmic inclusion bodies were first discovered (Bowden et al., 1991).

The issues on protein misfolding in the bacterial periplasm as well as heat-shock responses to extracytoplasmic stress have gained much attention, particularly during

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the past decade. Specific stress sensing and regulatory pathways of σ^E and Cpx, which are partially overlapping, have been identified (Alba and Gross, 2004; Connolly et al., 1997; Dartigalongue et al., 2001; Missiakas and Raina, 1997; Raivio and Silhavy, 1999). The responses to extracytoplasmic stress are driven by the synthesis of a variety of heat-shock proteins expressing protease activity that degrades misfolded proteins and/or chaperone activity that renatures misfolded proteins. As a practical application, periplasmic proteins with these protease and/or chaperone activities would be proper candidates for relieving extracytoplasmic stress for cells overexpressing foreign gene products.

Up to now, there are more than ten periplasmic proteases identified in *E. coli* (Miller, 1996). On the other hand, several periplasmic proteins, including DegP (Spiess et al., 1999), Skp (Bothmann and Pluckthun, 1998; Hayhurst and Harris, 1999), SurA (Lazar and Kolter, 1996), FkpA (Arie et al., 2001; Bothmann and Pluckthun, 2000), DsbC (Chen et al., 1999), and substrate-binding proteins (Richarme and Caldas, 1997), etc. have been identified to have chaperone activities and could play a role in the folding or targeting of extracytoplasmic proteins. Among these periplasmic proteases and chaperones, DegP and FkpA are two gene products in response to extracytoplasmic stress, such as heat shock or the presence of misfolded proteins, via the σ^E heat-shock regulon and Cpx two-component systems (Raivio and Silhavy, 1999).

DegP is a periplasmic heat-shock protein with both protease and chaperone activities (Spiess et al., 1999). It primarily acts as a serine protease for breakdown of aberrant periplasmic proteins arising upon extracytoplasmic stress (Kolmar et al., 1996; Strauch et al., 1989) and *degP* expression can be activated via both σ^E and Cpx pathways (Raivio and Silhavy, 1999). Coexpression of *degP* was applied to relieve extracytoplasmic stress upon the overexpression of several gene products, such as alkaline phosphatase (PhoA) (Kadokura et al., 2001), DsbA′-PhoA (Guigueno et al., 1997), MalS (Spiess et al., 1999), OmpF mutants (Misra et al., 2000), and maltose-binding protein (MBP or MalE) variants (Betton et al., 1996; Missiakas et al., 1996). FkpA is another periplasmic heat-shock protein with both *cis/trans* peptidyl-prolyl isomerase (PPIase) and chaperone activities (Arie et al., 2001; Ramm and Pluckthun, 2000; Saul et al., 2004). Expression of *fkpA* is activated via the σ^E pathway (Raivio and Silhavy, 1999) and is capable of reducing the σ^E-dependent response induced by misfolded proteins in the periplasm (Missiakas et al., 1996). It was also discovered that the production of functional recombinant antibody fragments could be improved by coexpression of *fkpA* (Bothmann and Pluckthun, 2000; Zhang et al., 2003).

In our laboratory, various biochemical and genetic strategies have been applied for the production of recombinant penicillin acylase (PAC), an important industrial enzyme for the production of several β-lactam antibiotics (Shewale and Sivaraman, 1989), in *E. coli*. PAC from *E. coli* serves as an interesting model protein for studying recombinant protein production because the formation of mature PAC in the periplasm involves a series of posttranslational steps, including translocation and periplasmic processing/folding steps, which are unique for prokaryotic proteins (Sizmann et al., 1990). The periplasmic processing mechanism is known to consist of various proteolytic steps via intramolecular autoproteolysis (Kasche et al., 1999; Sizmann et al., 1990). The formation of inclusion bodies, which are primarily composed of proPAC in the periplasm, was identified as an important obstacle for the overproduction of PAC in *E. coli* (Chou et al., 1999c; Scherrer et al., 1994; Sriubolmas et al., 1997). Hence, efforts have been directed toward tackling the issues of physiological stress and misfolding of proPAC in the periplasm.

Since the physiological stress and the bottleneck step limiting the overall production of PAC occur in the periplasm of *E. coli*, periplasmic heat-shock proteins potentially offer a promise for resolving this technical problem. We previously demonstrated that the presence of exogenous DegP significantly reduced the amount of periplasmic PAC inclusion bodies, suppressed the physiological stress, and enhanced the production of recombinant PAC in *E. coli* (Lin et al., 2001; Pan et al., 2003). DegP protease activity was primarily responsible for the improvement by degradation of abnormal proteins in the periplasm when *pac* was overexpressed (Pan et al., 2003). On the other hand, increasing the efficiency of the limiting step (i.e., posttranslational processing of proPAC) was recently demonstrated to be achievable by several cytoplasmic chaperone activities (Xu et al., 2005). The search for appropriate periplasmic chaperones that directly interact with periplasmic proPAC and suppress the periplasmic stress would provide hints for pinpointing the possible factors limiting *pac* overexpression. In this study, we investigated the genetic effect of *degP* and *fkpA*, and the physiological roles of their gene products upon *pac* overexpression. The study also demonstrated the potential applicability of genetic manipulation on cellular physiology to enhance recombinant protein production.

Materials and Methods

Bacterial Strains and Plasmids

Strains and plasmids used in the study are summarized in Table I and briefly described here. MDΔP7 was used as the host for the production of recombinant PAC. Compared to the parent strain of HB101, MDΔP7 could potentially have a higher *pac* translational and periplasmic processing efficiency for *pac* expression (Chou et al., 1999b). Molecular cloning was performed according to standard protocols (Sambrook et al., 1989) and HB101 was the host for cloning. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Polymerase chain reaction (PCR) was conducted in an automated thermal cycler (Amplitron II, Thermolyne, Dubuque, IA). Purification of plasmid DNA was performed using a spin-column kit purchased from

Table I. Summary of *E. coli* strains, plasmids, and oligonucleotides used in this study. For all the oligonucleotides, the designed restriction sites are underlined and the introduced mutations are italicized. DNA sequences in the lower case represent the overhanging region that is homologous to *degP* or *fkpA* for recombination.

Strain and DNA	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[#] (Boyer and Roulland-Dussoix, 1969)
MDΔP7	A penicillin-sensitive and 6-APA-resistant mutant derived from HB101	This lab (Chou et al., 1999b)
PDW172	MDΔP7, Δ*degP*::Cm^R	This study
PDW282	MDΔP7, Δ*degP*	This study
WU328	MDΔP7, Δ*fkpA*::Cm^R	This study
WU422	MDΔP7, Δ*fkpA*	This study
WU712	MDΔP7, Δ*degP*, Δ*fkpA*	This study
Plasmid		
pAR3	A P_{araB}-expression vector, Ori (pACYC184), Cm^R	J. Gutierrez (Perez-Perez and Gutierrez, 1995)
pARDegP	A *degP* expression vector, P_{araB}::*degP*, Ori (pACYC184), Cm^R	This lab (Pan et al., 2003)
pARDegP$_{S210A}$	A $degP_{S210A}$ expression vector, P_{araB}::$degP_{S210A}$, Ori (pACYC184), Cm^R	This lab (Pan et al., 2003)
pARFkpA	An *fkpA* expression vector, P_{araB}::*fkpA*, Ori (pACYC184), Cm^R	This study
pCP20	A helper plasmid for conducting FLP recombination, *flp*$^+$, Rep^{ts}, Ori (pSC101), Ap^R, Cm^R	W. Wackernagel (Cherepanov and Wackernagel, 1995)
pKD3	A template plasmid carrying the Cm^R gene flanked by FRT sites	B. L. Wanner (Datsenko and Wanner, 2000)
pKD46	A λ Red recombinase expression plasmid, P_{araB}::*gam*::*bet*::*exo*, Ori (pSC101), Ap^R	B. L. Wanner (Datsenko and Wanner, 2000)
pTrcKn99A	A P_{trc}-expression vector derived from pTrc99A, Ori (pBR322), Kn^R	This lab (Chou et al., 1999c)
pTrcKnPAC2902	A *pac* expression vector, P_{trc}::*pac*, Ori (pBR322), Kn^R	This lab (Chou et al., 1999c)
Oligonucleotide		
FKP1/FKP2	5′-*CT*CCATG*G*C*G*AAATCACTGTTTAAAGTAACGCT G-3′ 5′-CTAAATTAATACAGCGGAGGT*A*CCGCTTTC-3′ primer pair for amplification of *fkpA* for cloning	This study
PH1P1/PH2P2	5′-ctttagttcggaacttcaggctataaaacgaatctgaagaacacaGTGTA GGCTGGAGCTGCTTC-3′ 5′-tcacagattgtaaggagaacccttcccgttttcaggaaggggttATGGG AATTAGCCATGGTCC-3′ primer pair for amplification of the Cm^R fragment flanked by the *degP*-homologous regions for recombination	This study
P1/P2	5′-GTGTAGGCTGGAGCTGCTTC-3′ 5′-ATGGGAATTAGCCATGGTCC-3′ testing primer pair for *degP* genotype	This study
Ph1/Ph2	5′-CGGAACTTCAGGCTATAAAACGAATC-3′ 5′-GAACCCCTTCCCGTTTTCAGG-3′ testing primer pair for *degP* genotype	This study
PF1P1/PF2P2	5′-ccttctgataatagttaaccctggggtgagatgccccgatcctggGTGTA GGCTGGAGCTGCTTC-3′ 5′-ccggcctgtaataaaaaaaccgccgcctggtcaggcggcggttctATGG GAATTAGCCATGGTCC-3′ primer pair for amplification of the Cm^R fragment flanked by the *fkpA*-homologous regions for recombination	This study
P1/FKP2	5′-GTGTAGGCTGGAGCTGCTTC-3′ 5′-CTAAATTAATACAGCGGAGGTACCGCTTTC-3′ testing primer pair for *fkpA* genotype	This study
Pf1/Pf2	5′-ACCCTGGGGTGAGATGCCCC-3′ 5′-AAAAACCGCCGCCTGGTCAG-3′ testing primer pair for *fkpA* genotype	This study

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Clontech (Palo Alto, CA). Plasmid transformation was carried out using an electroporator (*E. coli* Pulser, Bio-Rad, Hercules, CA).

E. coli fkpA gene was PCR-amplified with the use of *pfu* DNA polymerase (Stratagene, La Jolla, CA), appropriate primers in Table I, and HB101 genomic DNA as the template. The PCR product flanked with *NcoI* and *KpnI* (0.91 kb) were purified and cloned into the corresponding restriction sites of pTrcKn99A. DNA sequencing was performed to ensure that no mutation was introduced in PCR. The *NcoI-KpnI* DNA fragment containing *fkpA* was subcloned into the *NcoI-KpnI* backbone of pAR3, which was treated by complete *KpnI*-digestion and partial *NcoI*-digestion, to form pARFkpA. The design of *NcoI* site in the sense primer resulted in the addition of Ala as the second amino acid of the signal peptides and this mutation appeared to be harmless for translocation. The construction of pARDegP and pARDegP$_{S210A}$ was described previously

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(Pan et al., 2003). pTrcKnPAC2902 contains the *pac* operon whose transcription is regulated by the *trc* promoter (Chou et al., 1999c). It has the pBR322 replication origin and, therefore, is compatible to various pAR3-derived plasmids containing the pACYC184 replication origin.

Since both DegP and FkpA have chaperone activities, the mutation on these corresponding genes was made by deleting the majority of the structural coding region to avoid any possible chaperone activities remained in the mutant gene product. MDΔP7 carrying wild-type *degP* and *fkpA* was used as the parent strain and the genotypes of various mutants are described in Table I. Construction of various *degP* and *fkpA* mutants was performed according to a developed protocol for *E. coli* chromosomal engineering (Datsenko and Wanner, 2000) and briefly described here. In essence, the Cm^R fragment flanked by the FRT sites and the DNA regions homologous to both termini of the target gene (*degP* or *fkpA*) was PCR amplified using pKD3 as the template, *pfu* DNA polymerase, and appropriate primers (PH1P1/PH2P2 for *degP* and PF1P1/PF2P2 for *fkpA*). The PCR product was appropriately cleaned, treated with DpnI, gel-recovered, and then electroporated into MDΔP7 harboring pKD46 using Cm as a marker. Plasmid pKD46 carries the phage λ Red recombinase for mediating the homologous recombination between the target gene on the genome and the transformed PCR-amplified fragment. The transformants were restreaked at 42°C and screened for those losing pKD46 (i.e., Ap^S clones). The Δ*degP*::Cm^R and Δ*fkpA*::Cm^R intermediate mutants were designated as PDW172 and WU328, respectively. The Cm^R fragment in the intermediate mutant was subsequently eliminated by Flp action, leaving behind a short nucleotide sequence with one FRT site as a deleted mutation (Δ*degP* or Δ*fkpA*). To remove the Cm^R gene in the genome of PDW172 and WU328, the cells were transformed with the helper plasmid of pCP20 that carries the *flp* gene. The transformants were restreaked at 42°C and screened for Cm^S and Ap^S clones. The Δ*degP*, Δ*fkpA*, and binary Δ*degP*/Δ*fkpA* mutants derived from MDΔP7 were designated as PDW282, WU422, and WU712, respectively. The genotype of the mutants was confirmed by colony-PCR using appropriate testing primer pairs in Table I. To do this, a single colony was resuspended in 20 μL DI water and heated at 95°C for 10 min. The suspension was centrifuged at 20,000*g* for 10 min and the supernatant was used as the template for colony-PCR.

Cultivation

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 LB medium, which was then incubated at 37°C on a rotary shaker at 220 rpm for approximately 15 h. The medium was supplemented with kanamycine (Kn) at 25 μg/mL or chloramphenicol (Cm) at 32 μg/mL when necessary. The seed culture at a volume of 10 mL was used to inoculate a lab-top bioreactor (Omni-Culture, VirTis, Gardiner, NY) containing 1 L working volume of LB medium. Isopropyl β-D-thiogalactopyranoside (IPTG) and arabinose were added to induce the synthesis of PAC and periplasmic chaperone (i.e., DegP or FkpA), respectively. 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, Columbus, OH), a pH controller (PC310, Suntex, Taipei, Taiwan), and two peristaltic pumps (101U/R, Watson Marlow, Falmouth, UK). The bioreactor was operated at 28°C and 500 rpm for approximately 50 h.

Analytical Methods

The culture sample was appropriately diluted with saline solution for measuring cell density in OD_{600} using a spectrophotometer (V-530, Jasco, Tokyo, Japan). For the preparation of cell extract, cells at an amount of 40 OD_{600}-units were centrifuged at 2°C and 6,000*g* for 5 min. The supernatant was assayed as the extracellular enzyme 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 as the intracellular enzyme activity. The PAC release level is defined as the percentage of total PAC activity detected in the extracellular medium. 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% SDS), and heated at 100°C for 5 min. The protein content of the pellet was analyzed as the insoluble fraction. All the analytical methods in this study, including microbiological screening of PAC-producing strains, PAC assay, SDS–polyacrylamide gel electrophoresis (SDS-PAGE), were performed as previously described (Chou et al., 1999c).

Results

Effect of Exogenous FkpA

The effect of exogenous FkpA on *pac* overexpression can be observed by comparing the culture performance for the two strains of MDΔP7 harboring pTrcKnPAC2902 and MDΔP7 harboring pTrcKnPAC2902 and pARFkpA, and the results are summarized in Figure 1. For the control experiment using MDΔP7 harboring pTrcKnPAC2902, the final cell density reached 3.7 OD_{600} with a specific PAC activity of 334 U/L/OD_{600}. Up to 21% of PAC activity was detected in

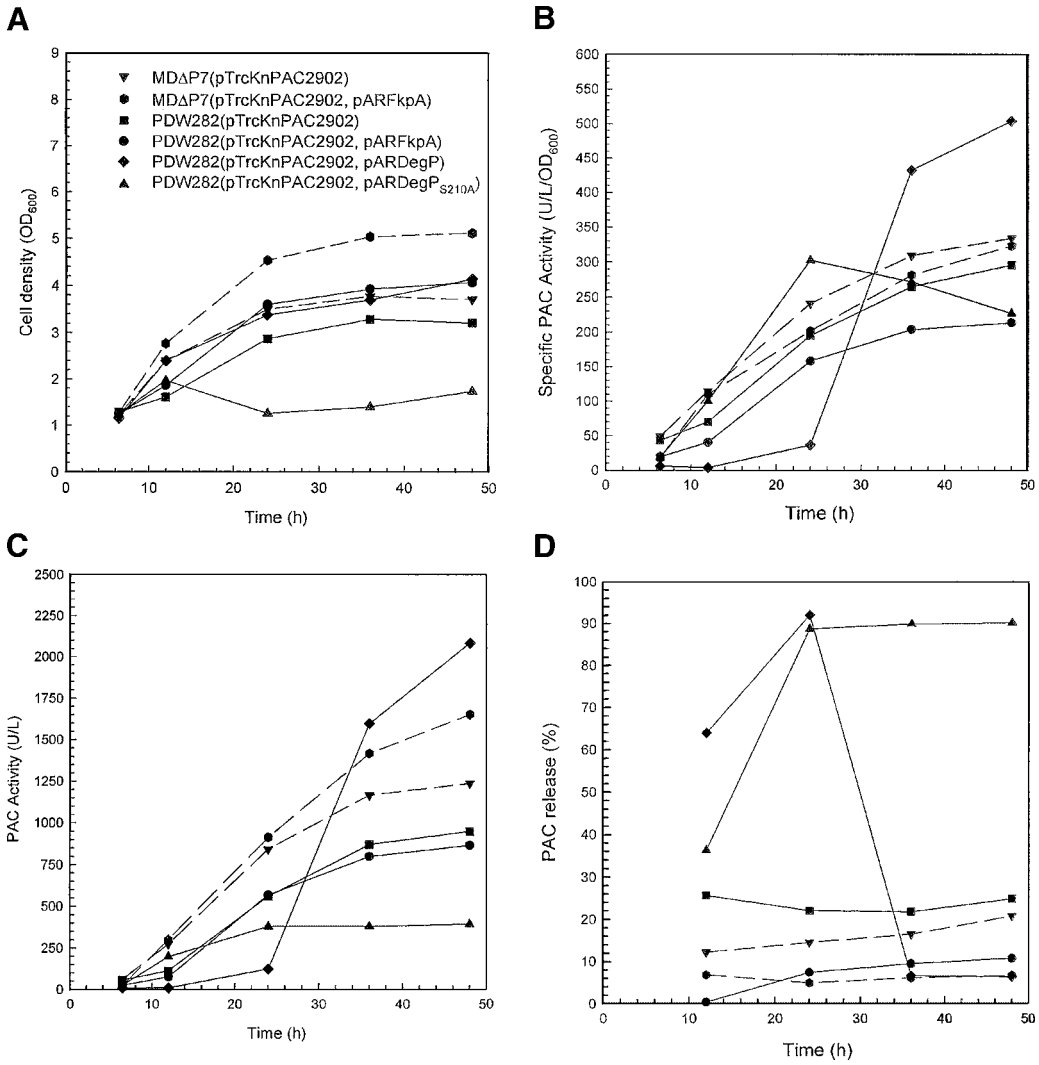

Figure 1. Effect of Δ*degP* mutation and exogenous periplasmic chaperones on the production of PAC: Time profiles of cell density (**panel A**), specific PAC activity (**panel B**), volumetric PAC activity (**panel C**), and PAC release level (**panel D**) for the cultures of various host/vector systems are shown. All the cultures were supplemented with 0.1 mM IPTG for induction of PAC synthesis when the first sample was taken (at OD_{600} of 1.0∼1.3). For the cultures of binary-plasmid host, arabinose at 0.05 g/L was supplemented for induction of chaperone synthesis when the first sample was taken.

the extracellular medium, implying that some cells were lysed due to a relatively stressful physiological condition. Coexpression of *fkpA* resulted in 40% increase in the final cell density (5.1 vs. 3.7 OD_{600}) without affecting the final *pac* expression level (323 vs. 334 U/L/OD_{600}). In addition, PAC release level was significantly reduced (6.6% vs. 21%), implying that the physiological stress was partially alleviated. This improvement in the production of PAC is rather similar to that caused by *degP* coexpression (Pan et al., 2003), except that *pac* expression level was not increased and the amount of PAC inclusion bodies remained relatively unchanged, compared to those of the control experiment (see below).

Effect of the *ΔdegP* Mutation

The effect of Δ*degP* mutation on *pac* overexpression can be observed by comparing culture performance for the parent strain of MDΔP7 and the Δ*degP* mutant of PDW282 harboring various plasmids, and the results are summarized in Figure 1. For the Δ*degP* mutant of PDW282 harboring pTrcKnPAC2902, cell growth rate was significantly lower than that of the control experiment using MDΔP7 harboring pTrcKnPAC2902, particularly during the exponential phase, though the final cell density only had a slight decrease compared to the control experiment (3.2 vs. 3.7 OD_{600}). The culture also had a slight decrease in the specific PAC activity

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compared to the control experiment (296 vs. 334 U/L/OD_{600}). Cell physiology was however not seriously affected by the Δ*degP* mutation since PAC release level remained approximately the same as that of the control experiment (25% vs. 21%). The defect in cell growth was complemented by the presence of exogenous DegP, as shown in the culture performance for PDW282 harboring pTrcKnPAC2902 and pARDegP. Not only the cell growth rate was recovered to the level of the parent strain of MDΔP7 harboring pTrcKnPAC2902, but also the specific PAC activity was significantly increased (504 vs. 334 U/L/OD_{600}). In addition, cell physiology was improved since PAC release level decreased significantly (6% vs. 25%). However, the defect in culture performance was not complemented by the presence of exogenous $DegP_{S210A}$, which is a DegP mutant lacking the protease activity but still retaining the chaperone activity. Both cell growth rate and *pac* expression level for PDW282 harboring pTrcKnPAC2902 and $pARDegP_{S210A}$ decreased, compared to those of PDW282 harboring pTrcKnPAC2902 (1.7 vs. 3.2 OD_{600} and 227 vs. 295 U/L/OD_{600}). In addition, cell physiology deteriorated significantly and PAC release level highly increased to 90%. The growth defect caused by the Δ*degP* mutation was partially complemented by the presence of exogenous FkpA, as shown in the culture performance for PDW282 harboring pTrcKnPAC2902 and pARFkpA. Compared to PDW282 harboring pTrcKnPAC2902, the final cell density had a significant increase (4.1 vs. 3.2 OD_{600}). Cell physiology was also improved since PAC release level decreased significantly (11% vs. 25%). However, it appears that *pac* expression level was diluted by the increased cell density.

Effect of the Δ*fkpA* Mutation

The effect of Δ*fkpA* mutation on *pac* overexpression can be observed by comparing the culture performance for the parent strain of MDΔP7 and the Δ*fkpA* mutant of WU422 harboring various plasmids and the results are summarized in Figure 2. Compared to the control experiment using MDΔP7 harboring pTrcKnPAC2902, the final cell density for the culture of WU422 harboring pTrcKnPAC2902 had a significant decrease (1.7 vs. 3.7 OD_{600}) with approximately the same *pac* expression (331 vs. 334 U/L/OD_{600}). Cell physiology was seriously affected by the Δ*fkpA* mutation since PAC release level significantly increased, compared to that of the control experiment (63% vs. 21%). The defect in cell growth was complemented by the presence of exogenous FkpA, as shown in the culture performance for WU422 harboring pTrcKnPAC2902 and pARFkpA. The final cell density was significantly increased (5.0 vs. 1.7 OD_{600}) while approximately the same *pac* expression level was maintained (307 vs. 331 U/L/OD_{600}). In addition, cell physiology was significantly improved since little PAC was released extracellularly (4% vs. 63%). The growth defect was also complemented by the presence of exogenous DegP, as shown in the culture performance for WU422 harboring pTrcKnPAC2902 and pARDegP, which had a significant increase not only in the final cell density (4.3 vs. 1.7 OD_{600}) but also *pac* expression level (514 vs. 331 U/L/OD_{600}). In addition, cell physiology was significantly improved as PAC release level was extremely low (3% vs. 63%). Again, the defect in culture performance was not complemented by the presence of exogenous DegP mutant, $DegP_{S210A}$. Growth arrest was still observed for the culture of WU422 harboring pTrcKnPAC2902 and $pARDegP_{S210A}$ and *pac* expression level became even lower, compared to WU422 harboring pTrcKnPAC2902 (1.8 vs. 1.7 OD_{600} and 215 vs. 331 U/L/OD_{600}). In addition, cell physiology deteriorated significantly as PAC release level highly increased up to 89%.

Effect of the Binary Mutation of Δ*degP*/Δ*fkpA*

The effect of the Δ*degP*/Δ*fkpA* binary mutation on *pac* overexpression can be observed by comparing the culture performance for the parent strain of MDΔP7 and the Δ*degP*/Δ*fkpA* mutant of WU712 harboring various plasmids and the results are summarized in Figure 3. Compared to the control experiment using MDΔP7 harboring pTrcKnPAC2902, the binary mutation of Δ*degP*/Δ*fkpA* seriously inhibited cell growth (1.1 vs. 3.7 OD_{600}) even though *pac* expression appeared to be unaffected (421 vs. 334 U/L/OD_{600}), resulting in a significant decrease in the volumetric PAC activity (459 vs. 1238 U/L). Cell physiology was seriously affected as PAC release level increased to 95%. The growth defect was complemented by the presence of either DegP or FkpA. The final cell density for the cultures of WU712 harboring pTrcKnPAC2902 and pARDegP and WU712 harboring pTrcKnPAC2902 and pARFkpA reached 4.3 and 5.0 OD_{600}, respectively, by maintaining a relatively high *pac* expression level (362 and 359 U/L/OD_{600}, respectively). Such culture performance in PAC production is comparable to that for the control experiment of MDΔP7 harboring pTrcKnPAC2902. Moreover, cell physiology was improved upon coexpression of *degP* or *fkpA* since PAC release level was reduced to 2% and 8%, respectively.

Genetic Effect on the Formation of PAC Inclusion Bodies

Genetic effect of *degP* and *fkpA* on the formation of PAC inclusion bodies is summarized in Figure 4. For the control experiment using MDΔP7 harboring pTrcKnPAC2902, formation of inclusion bodies was observed though the production of PAC was effective (lane C1). The inclusion bodies were primarily composed of proPAC in the periplasm according to previous reports (Chou et al., 1999a; Scherrer et al., 1994; Sriubolmas et al., 1997). Compared to the control experiment, the amount of PAC inclusion bodies remained relatively unchanged upon coexpression of *fkpA* (lane C2) though the overall culture performance was significantly improved (Fig. 1). In addition, the amount of PAC inclusion bodies was not

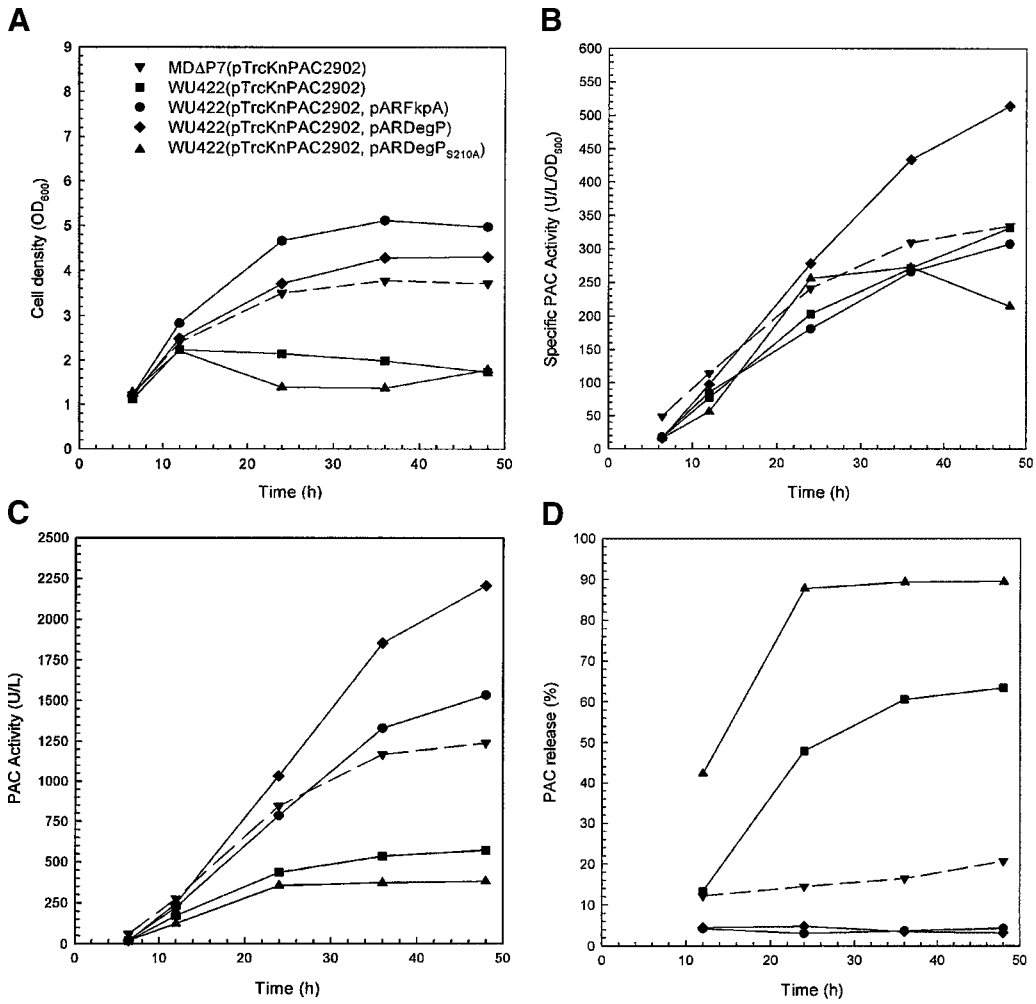

Figure 2. Effect of Δ*fkpA* mutation and exogenous periplasmic chaperones on the production of PAC: Time profiles of cell density (**panel A**), specific PAC activity (**panel B**), volumetric PAC activity (**panel C**), and PAC release level (**panel D**) for the cultures of various host/vector systems are shown. All the cultures were supplemented with 0.1 mM IPTG for induction of PAC synthesis when the first sample was taken (at OD_{600} of 1.0∼1.3). For the cultures of binary-plasmid host, arabinose at 0.05 g/L was supplemented for induction of chaperone synthesis when the first sample was taken.

affected by the Δ*degP* mutation (lane 1). Coexpression of *fkpA* even resulted in an increase in the amount of PAC inclusion bodies under this genetic background (lane 2). On the other hand, these inclusion bodies were cleaned up by exogenous DegP (lane 3), but not by the mutant of $DegP_{S210A}$ (lane 4). The Δ*fkpA* mutation also resulted in an increase in the amount of PAC inclusion bodies (lane 5) and this phenotypic increase was suppressed by coexpression of *fkpA* under this genetic background (lane 6). Note that these inclusion bodies were not completely cleaned up, suggesting that exogenous FkpA only removed the extra amount of PAC inclusion bodies caused by the Δ*fkpA* mutation. Similar to the Δ*degP* host/vector system, these inclusion bodies were completely removed by exogenous DegP (lane 7), but not by the mutant of $DegP_{S210A}$ (lane 8). The Δ*degP*/Δ*fkpA* binary mutation also resulted in a significant increase in the amount of PAC inclusion bodies (lane 9). Similar to the Δ*fkpA* host/vector system, these inclusion bodies were partially and completely removed by exogenous FkpA and DegP, respectively (lanes 10 and 11).

Discussion

It has been well recognized that the overexpression of foreign gene products in *E. coli* tends to pose physiological stress on the host/vector system. Strategies for improving recombinant protein production have been developed based on alleviating the stressful conditions induced by high-level gene expression (Barth et al., 2000; March et al., 2002; Spada et al., 2002). Based on the previous results (Chou et al., 1999c; Lin et al., 2001; Pan et al., 2003; Scherrer et al., 1994;

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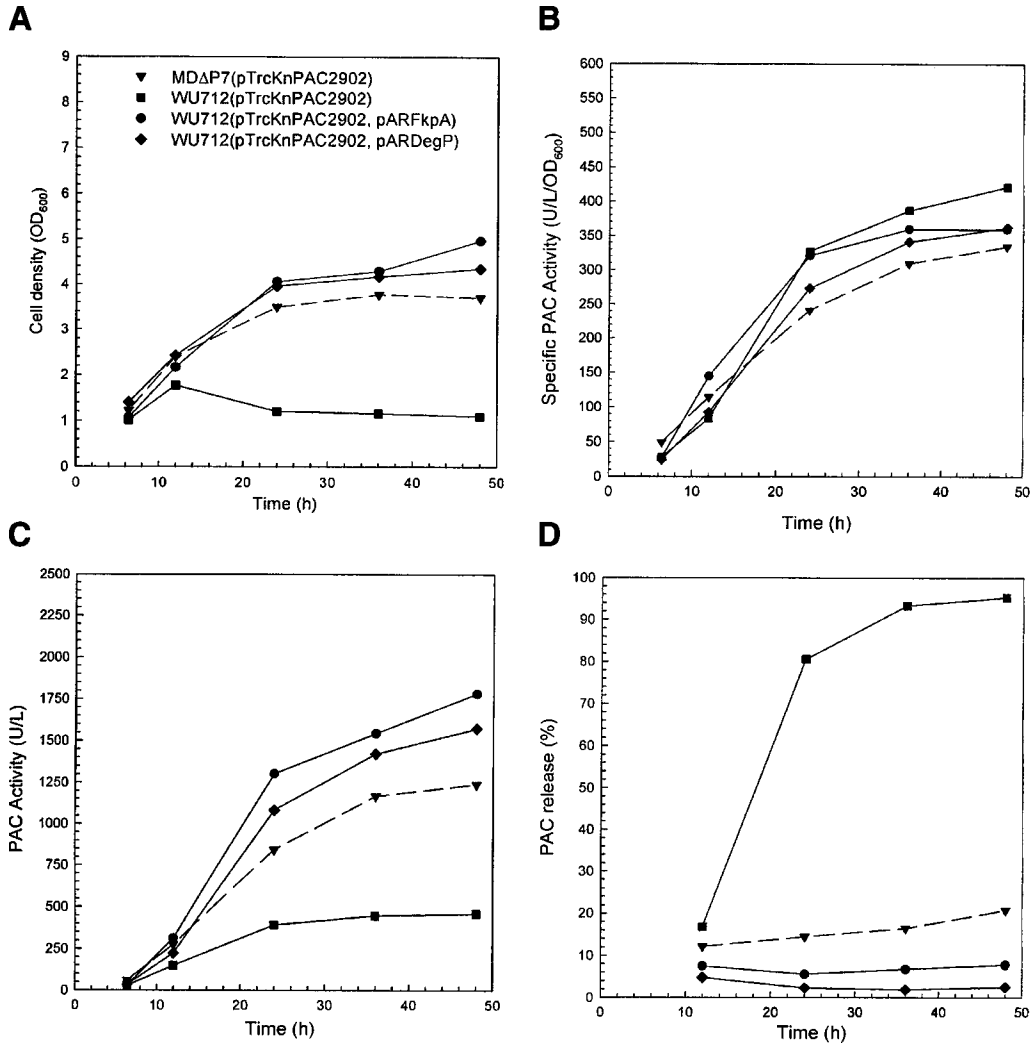

Figure 3. Effect of Δ*degP*/Δ*fkpA* mutation and exogenous periplasmic chaperones on the production of PAC: Time profiles of cell density (**panel A**), specific PAC activity (**panel B**), volumetric PAC activity (**panel C**), and PAC release level (**panel D**) for the cultures of various host/vector systems are shown. All the cultures were supplemented with 0.1 mM IPTG for induction of PAC synthesis when the first sample was taken (at OD_{600} of 1.0∼1.3). For the cultures of binary-plasmid host, arabinose at 0.05 g/L was supplemented for induction of chaperone synthesis when the first sample was taken.

Sriubolmas et al., 1997), several technical issues need to be addressed for the high-level production of recombinant PAC in *E. coli*. First, the production of PAC was limited by the initiation step of periplasmic processing since proPAC was the major aggregative species upon *pac* overexpression. The periplasmic processing machinery was overwhelmed by proPAC transiently formed in an excess amount. Folding of proPAC in the periplasm was not properly carried out for subsequent autoproteolysis (Kasche et al., 1999), possibly due to an increased level of the local protein concentration. This limitation can possibly be resolved by periplasmic chaperones. Second, cell physiology was seriously affected as a result of extracytoplasmic stress, such as protein misfolding and failure of degradation of misfolded proteins in the periplasm upon *pac* overexpression. This limitation can possibly be resolved by periplasmic proteases and/or chaperones.

Several periplasmic heat-shock proteins can express the protease activity for degrading misfolded proteins and/or the chaperone activity for renaturing misfolded proteins. Among them, DegP exhibits protease or chaperone activity in the periplasm using temperature as an environmental switch; namely the protease and chaperone activities dominate under high and low temperatures, respectively (Spiess et al., 1999). On the other hand, FkpA plays an active role as a folding catalyst or chaperone in the periplasm via decreasing the σ^E-dependent response constitutively induced by misfolded proteins (Missiakas et al., 1996). The two periplasmic heat-shock proteins appear to be effective for enhancing the production of PAC via

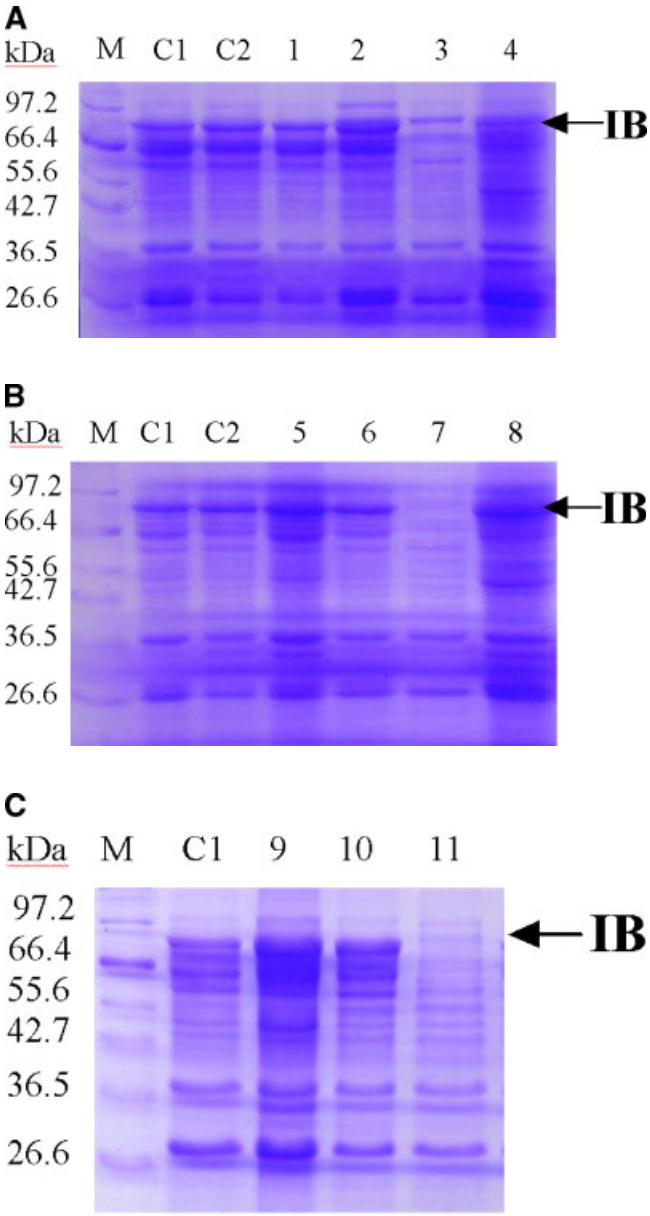

Figure 4. **Panel A**, **B**, and **C** are the results of SDS–PAGE analysis of the protein content of the insoluble fraction for the last sample of various cultures in Figures 1–3, respectively. **Lane M**, protein markers; C1, MDΔP7 harboring pTrcKnPAC2902 as the control experiment; C2, MDΔP7 harboring pTrcKnPAC2902 and pARFkpA; 1, PDW282 harboring pTrcKnPAC2902; 2, PDW282 harboring pTrcKnPAC2902 and pARFkpA; 3, PDW282 harboring pTrcKnPAC2902 and pARDegP; 4, PDW282 harboring pTrcKnPAC2902 and pARDegP$_{S210A}$; 5, WU422 harboring pTrcKnPAC2902; 6, WU422 harboring pTrcKnPAC2902 and pARFkpA; 7, WU422 harboring pTrcKnPAC2902 and pARDegP; 8, WU422 harboring pTrcKnPAC2902 and pARDegP$_{S210A}$; 9, WU712 harboring pTrcKnPAC2902; 10, WU712 harboring pTrcKnPAC2902 and pARFkpA; 11, WU712 harboring pTrcKnPAC2902 and pARDegP. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

suppression of physiological toxicity caused by *pac* overexpression. Our previous reports indicate that the presence of exogenous DegP significantly enhanced the production of recombinant PAC and decreased the amount of PAC inclusion bodies in the periplasm (Lin et al., 2001; Pan et al., 2003). It was discovered that DegP primarily functioned as a protease to improve cell physiology by preventing the misfolding and aggregation of various periplasmic proteins (including PAC precursors) upon *pac* overexpression (Pan et al., 2003). We also speculated that chaperone activity might be capable of improving the culture performance. The current result that the presence of exogenous FkpA, which is a periplasmic chaperone, can alleviate physiological stress and subsequently improve the production of PAC (Fig. 1) supports the hypothesis. To further identify the role that the two proteins play upon *pac* overexpression, various strains with the mutation(s) on *degP* and/or *fkpA* were used as the host for systematic investigation of the mutational effect on the production of PAC.

It appears that the Δ*degP* mutation resulted in a decrease in cell growth rate, particularly during the exponential phase, though PAC production was slightly affected. The result suggests that DegP is not directly involved in periplasmic processing of proPAC to form mature PAC. The decrease in cell-growth rate could possibly result from the defect in certain house-keeping function related to DegP and this phenotypic defect was complemented by exogenous DegP. The Δ*degP* growth defect was also complemented by exogenous FkpA (Fig. 1). The role that DegP played for the significant increase in the specific PAC activity, reduction in the amount of inclusion bodies, and improvement in cell physiology was previously discussed (Pan et al., 2003). Although the Δ*degP* mutation did not much increase PAC release level upon *pac* overexpression, the physiological defect was in fact improved by coexpression of either *degP* or *fkpA*. The result suggests that DegP and FkpA might have certain overlapping function for suppression of the toxicity caused by *pac* overexpression. It is noteworthy that the genetic evidence for parallel pathways of chaperone activity in the periplasm of *E. coli* has been provided (Rizzitello et al., 2001) since both DegP and FkpA have chaperone activities. However, DegP$_{S210A}$, which lacks the protease activity but still retains the chaperone activity (Skorko-Glonek et al., 1995; Spiess et al., 1999), failed to show any suppressive effect, implying that DegP protease activity was critical for the suppression. It is surprising to observe that the presence of DegP$_{S210A}$, a protease-deficient mutant derived based on such single-amino-acid mutation, posed a serious toxicity on cellular physiology even though its chaperone activity was retained. More study will be required to characterize such deleterious effect on the production of PAC.

Similarly, the Δ*fkpA* mutation resulted in a growth defect and deterioration in cell physiology. However, *pac* expression was slightly affected (Fig. 2), suggesting that FkpA might not be directly involved in periplasmic processing of proPAC to form mature PAC. The growth defect might be caused by the loss of certain house-keeping function related to FkpA and could be complemented by the presence of exogenous FkpA. In addition, coexpression of *degP* not only recovered the growth defect but also significantly increased *pac* expression level. The result supports the argument that

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DegP and FkpA shared certain overlapping function for improving cell physiology upon *pac* overexpression.

The growth arrest caused by the binary mutation of Δ*degP*/Δ*fkpA* was rather unsurprising as both single mutations resulted in growth defect to some extent. The result that *pac* expression level was slightly affected supports the argument that neither DegP nor FkpA was directly involved in the periplasmic processing of proPAC. The defect in culture performance could be complemented by the presence of exogenous DegP or FkpA. It was interesting to observe that, unlike the previous cases in which DegP always outperformed FkpA in terms of resulting in higher *pac* expression levels when presenting extrachromosomally, the extents of the improvement mediated by the presence of the two periplasmic chaperones were approximately the same under this genetic background (i.e., Δ*degP*/Δ*fkpA*). The result suggests that the binary mutation of Δ*degP*/Δ*fkpA* could have synergistically induced a defect in *pac* expression such that the significant improving effect due to the presence of exogenous DegP was no longer observed. The result again supports the argument that DegP and FkpA could share certain overlapping function mediating the enhancement in *pac* expression.

Although both exogenous DegP and FkpA appeared to be effective in enhancing the production of PAC, the mechanisms leading to the respective improvement were believed to be different. Coexpression of *degP*: (1) prevented misfolding of proPAC, in light of a reduction in the amount of PAC inclusion bodies; (2) shifted PAC synthesis flux from the non-productive pathway to the productive one, in light of an increase in *pac* expression level; (3) improved cell physiology, in light of an enhanced cell growth and less cell lysis. However, only cellular physiology was improved upon coexpression of *fkpA*. FkpA neither reduced the amount of PAC inclusion bodies nor increased the *pac* expression level under any genetic backgrounds.

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