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Roles of DegP in Prevention of Protein Misfolding in the Periplasm upon Overexpression of Penicillin Acylase in *Escherichia coli*

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Enhancement of the production of soluble recombinant penicillin acylase in *Escherichia coli* via coexpression of a periplasmic protease/chaperone, DegP, was demonstrated. Coexpression of DegP resulted in a shift of in vivo penicillin acylase (PAC) synthesis flux from the nonproductive pathway to the productive one when *pac* was overexpressed. The number of inclusion bodies, which consist primarily of protein aggregates of PAC precursors in the periplasm, was highly reduced, and the specific PAC activity was highly increased. DegP was a heat shock protein induced in response to *pac* overexpression, suggesting that the protein could possibly suppress the physiological toxicity caused by *pac* overexpression. Coexpression of $DegP_{S210A}$, a DegP mutant without protease activity but retaining chaperone activity, could not suppress the physiological toxicity, suggesting that DegP protease activity was primarily responsible for the suppression, possibly by degradation of abnormal proteins when *pac* was overexpressed. However, a shortage of periplasmic protease activity was not the only reason for the deterioration in culture performance upon *pac* overexpression because coexpression of a DegP-homologous periplasmic protease, DegQ or DegS, could not suppress the physiological toxicity. The chaperone activity of DegP is proposed to be another possible factor contributing to the suppression.

The well-known genetic information of *Escherichia coli* and successful applications of recombinant DNA technology make it possible for a variety of attempts with genetic engineering techniques to overproduce recombinant proteins. Upon performing the cultivation for recombinant protein production, there are two primary goals: high-cell-density cultivation and high-level gene expression. Culture performance can be optimized when the two goals are achieved simultaneously. High-cell-density culture can be obtained by fed-batch cultivation (48), in which concentrated medium is fed gradually into the bioreactor. The primary concern of this operation is developing an optimum feeding strategy, based on which cells can be maintained in a high-energy state for enhancing gene expression while cells are growing.

On the other hand, various genetic strategies have been developed for high-level gene expression (24). The use of strong promoters (e.g., *tac*, *trc*, and T7) for regulation of gene expression has been successfully applied to enhance recombinant protein production by improving transcriptional efficiency and perhaps translational efficiency as well (35). However, it is a common problem that insoluble protein aggregates, known as inclusion bodies, tend to accumulate inside the cells upon overproduction of gene products (14, 44). While the mechanism of inclusion body formation is not completely understood, it is believed that the overexpressed gene products cannot be suitably processed by folding modulators to develop a proper protein structure (46). For proteins destined to be exported, the protein formation mechanism would be more complicated, and the efficiency of translocation, posttranslocational folding, processing, and targeting becomes important. In principle, the precursors, intermediates, or final gene products can possibly form inclusion bodies in the cytoplasm and/or periplasm upon gene overexpression (4). This raises an important issue that, in addition to improving the efficiency of each gene expression step (i.e., transcription, translation, and posttranslational steps), balanced protein synthesis flux throughout these steps should be properly maintained to avoid the accumulation of any protein species upon the overproduction of recombinant proteins.

Within the past decade, the issues of protein misfolding in the bacterial periplasm and heat shock responses to extracytoplasmic stresses began to gain attentions. A specific periplasmic heat shock regulon of σ^E, which is similar to the σ^H heat shock regulon in the cytoplasm, has been identified in *E. coli* (9, 29, 32). Just as occurs in the cytoplasm, the heat shock responses to extracytoplasmic stresses were driven by synthesis of a variety of heat shock proteins expressing protease activity that degrades misfolded proteins and/or chaperone activity that renatures misfolded proteins. From the viewpoint of application, periplasmic proteins with these protease and/or chaperone activities would be proper candidates for relieving extracytoplasmic stresses when cells are overexpressing foreign gene products.

More than 10 periplasmic proteases have been identified in *E. coli* (25). On the other hand, several periplasmic proteins, including DegP (also known as HtrA or Do) (42), Skp (11), SurA (20), and FkpA (2), have been identified as having chaperone activity 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 induced in response to extracytoplasmic stresses, such as heat shock or the presence of misfolded proteins, via the σ^E heat shock regulon and Cpx two-component systems (32).

DegP is perhaps the only periplasmic heat shock protein with both protease and chaperone activities. It has an inducible

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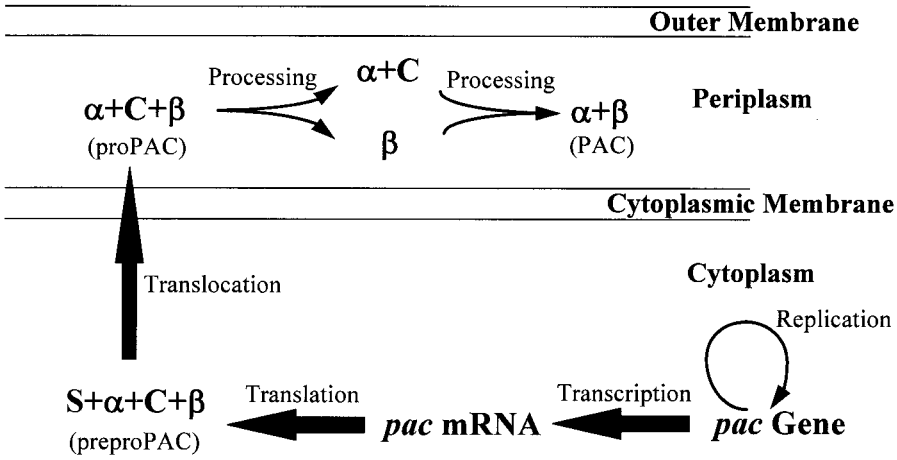


FIG. 1. Synthesis and maturation of PAC in *E. coli*. The structural *pac* gene from *E. coli* ATCC 11105 encodes a 95-kDa polypeptide precursor (preproPAC) composed of, from the N terminus to the C terminus, a signal peptide (S), α subunit (α), connecting peptide (C), and β subunit (β). The signal peptide directs the export of preproPAC into the periplasm and is removed after translocation. Another type of PAC precursor, proPAC, 92 kDa, is formed in the periplasm. Periplasmic processing, which involves a series of proteolytic steps, follows to remove the connecting peptide, and the two subunits (α at 24 kDa and β at 62 kDa) become available for assembly of mature PAC.

serine protease activity for breakdown of aberrant periplasmic proteins arising upon extracytoplasmic stresses (17, 45). Another uncommon feature of DegP is that this protein can exhibit either protease or chaperone activity with temperature as the regulatory switch (42). With these multiple functions, DegP is known to be involved in relieving extracytoplasmic stresses upon overexpression of several gene products, including alkaline phosphatase (PhoA) (13), DsbA′-PhoA (10), MalS (42), and maltose-binding protein (MalE) variants (3, 28).

We previously demonstrated that the presence of exogenous DegP could enhance the production of recombinant penicillin acylase (PAC), an important industrial enzyme for the production of many β-lactam antibiotics (37), in *E. coli* (21). The formation of mature PAC in the periplasm involves a series of posttranslational steps, including translocation and periplasmic processing/folding steps, which are unusual for prokaryotic proteins (Fig. 1) (38). The periplasmic processing mechanism is known to consist of various proteolytic steps via intramolecular autoproteolysis (15, 38). The formation of inclusion bodies, which are composed primarily of PAC precursors in the periplasm, was recently identified as an important obstacle to the overproduction of PAC in *E. coli* (8, 36, 43). Hence, efforts have been directed to reducing the number of periplasmic PAC inclusion bodies.

In this study, we provide evidence that DegP functions primarily as a protease to improve cell physiology by preventing the misfolding and aggregation of periplasmic PAC precursors when *pac* is overexpressed. Cultivation performance for the production of recombinant PAC was significantly enhanced due to improved cell physiology as well as simultaneous increases in the *pac* gene expression level and culture cell density.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in the study are summarized in Table 1 and briefly described here. MDΔP7 was used as the host for the production of recombinant PAC. Compared to the parent strain, *E. coli* HB101, MDΔP7 could potentially have higher *pac* translational and periplasmic processing efficiency for *pac* expression (7). MC4100λφ[*htrA-lacZ*] contains a single copy of the P_{degP}::*lacZ* transcriptional fusion at the λ phage attachment site of the chromosome (12). Molecular cloning was performed according to standard protocols (34), and HB101 was the host for cloning. Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.). PCR was conducted in an automated thermal cycler (Amplitron II; Thermolyne, Dubuque, Iowa). Purification of plasmid DNA was performed with a spin column kit purchased from Clontech (Palo Alto, Calif.) or Viagen (Taipei, Taiwan). Plasmid transformation was carried out with an electroporator (*E. coli* Pulser; Bio-Rad, Hercules, Calif.).

E. coli genes *degP*, *degQ*, and *degS* were amplified by PCR with *PFU* DNA polymerase (Stratagene, La Jolla, Calif.) and appropriate primers (Table 1). The PCR templates were pKS12 (45) for *degP* and HB101 genomic DNA for *degQ* and *degS*. The PCR products flanked with *Nco*I and *Kpn*I sites (1.55, 1.44, and 1.24 kb for *degP*, *degQ*, and *degS*, respectively) were purified and cloned into the corresponding restriction sites of pTrcKn99A. DNA sequencing of the PCR-cloned genes was performed with pTrcKn99A-derived vectors. The *Nco*I-*Kpn*I DNA fragments containing *degP*, *degQ*, and *degS* were subcloned into the *Nco*I-*Kpn*I backbone of pAR3, which had been treated by complete *Kpn*I digestion and partial *Nco*I digestion, to form pARDegP, pARDegQ, and pARDegS, respectively. The design of the *Nco*I site in the sense primers resulted in changes in the second amino acid of the signal peptides of Lys→Ala for DegP and DegQ and Phe→Val for DegS, and these mutations did not appear to affect translocation.

In vitro site-directed mutagenesis was conducted with the QuickChange kit (Stratagene, La Jolla, Calif.) according to the manufacturer's protocol. pARDegP was the mutagenesis template, and PSA/APSA were the mutagenesis primers; they contain a silent mutation with an extra *Nar*I site for screening purposes. pTrcKnPAC2902 contains the *pac* operon, whose transcription is regulated by the *trc* promoter (8). It has the pBR322 replication origin and is therefore compatible with various pAR3-derived plasmids carrying the 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 of NaCl, 5 g of Bacto yeast extract, 10 g of Bacto tryptone, and 15 g of Bacto agar per liter). 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 kanamycin at 25 μg/ml or chloramphenicol at 32 μg/ml when necessary. The seed culture at a volume of 10 ml was used to inoculate a table-top bioreactor (Omni-Culture; VirTis, Gardiner, N.Y.) containing 1 liter of working volume of LB medium. Unless otherwise specified, isopropyl-β-D-thiogalactopyranoside (IPTG) and arabinose were added simultaneously to induce the synthesis of PAC and protease (DegP, DegQ, or DegS), respectively. The culture was supplemented with 10 μl of antifoam 289 per liter (Sigma, St. Louis, Mo.) to avoid excessive

TABLE 1. Strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Relevant genotype or phenotype[a]	Source or reference
E. coli		
HB101	F$^-$ *hsdS20 leuB6 recA13 ara-14 proA2 lacY1 thi-1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	CCRC[b] (5)
MDΔP7	Penicillin-sensitive and 6-APA-resistant mutant derived from HB101	This lab (7)
MC4100λϕ [*htrA-lacZ*]	MC4100 λ[P_{degP}::*lacZ*]	K. Makino (12)
Plasmid		
pAR3	A P_{araB} expression vector, *ori* pACYC184, Cm^r	J. Gutierrez (31)
pARDegP	Expression vector containing *degP* fused with *araB* promoter, *ori* pACYC184, Cm^r	This study
pARDegP$_{S210A}$	Expression vector containing *degP*$_{S210A}$ fused with *araB* promoter, *ori* pACYC184, Cm^r	This study
pARDegQ	Expression vector containing *degQ* fused with *araB* promoter, *ori* pACYC184, Cm^r	This study
pARDegS	Expression vector containing *degS* fused with *araB* promoter, *ori* pACYC184, Cm^r	This study
pKS12	Expression vector containing *degP* gene, *ori* pACYC184, Cm^r	J. Beckwith (45)
pTrcKn99A	P_{trc} expression vector derived from pTrc99A, *ori* pBR322, Km^r	This lab (8)
pTrcKnPAC2902	Expression vector containing *pac* operon fused with *trc* promoter, *ori* pBR322, Km^r	This lab (8)
Oligonucleotides		
PA and PB	5′-AAAT*CCATGG*CAAAAACCACATTAGCACTG-3′ and 5′-CGTAA*GGTACC*GCATTGTGCAA-3′; primer pair for amplification of *degP*	This study
PC and PD	5′-GGAA*CCATGG*CAAAACAAACCCAG-3′ and 5′-GAACGGCAGCAG*GGTACC*ACG-3′; primer pair for amplification of *degQ*	This study
PE and PF	5′-GCCTCCA*CCATGG*TTGTGAAGCTCTTAC-3′ and 5′-AAATCTATC*GGTACC*CTGAGCGCA-3′; primer pair for amplification of *degS*	This study
PSA and APSA	5′-CCGTGGTAAC*G*CCGGT*GGCGCC*CTGGTTAAC-3′ and 5′-GTTAACCAG*GGCGCC*ACCGGCGTTACCACGG-3′; primer pair for site-directed mutagenesis of *degP*	This study

[a] Designed restriction sites are underlined and introduced mutations are in italics. 6-APA, 6-aminopenicillanic acid.
[b] Culture Collection & Research Center, Taiwan, ROC.

foaming. Filter-sterilized air at 1.5 liters/min was purged into the culture for aeration. The culture pH was held at 7.0 ± 0.1 by adding 3 N NaOH or 3 N HCl with a combined pH electrode (Mettler-Toledo, Berne, Switzerland), a pH controller (PC310; Suntex, Taipei, Taiwan), and two peristaltic pumps (101U/R; Watson Marlow, Falmouth, United Kingdom). 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 as the optical density at 600 nm (OD_{600}) with a spectrophotometer (V-530; Jasco, Tokyo, Japan). For the preparation of cell extract, 40 OD_{600} units of cells were centrifuged at 2°C and 6,000 × *g* for 5 min. The supernatant was assayed for 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 with an ultrasonic processor (Sonics & Materials, Danbury, Conn.) and then centrifuged at 2°C and 20,000 × *g* for 20 min. The supernatant containing soluble proteins was assayed for the intracellular enzyme 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 [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), and immunological analysis (Western blotting), were performed as described previously (8). β-Galactosidase was assayed as described previously (26). All assays were conducted in duplicate.

RESULTS

Effect of DegP coexpression on *pac* overexpression. For the control experiments with MDΔP7 harboring pTrcKnPAC2902 and MDΔP7 harboring pTrcKnPAC2902 and pAR3, the culture performance in *pac* overexpression is summarized in Fig. 2. In all cases, formation of inclusion bodies was observed (Fig. 3), though the production of PAC was effective. The inclusion bodies were primarily composed of proPAC in the periplasm, as described in previous reports (6, 36, 43). The presence of exogenous DegP resulted in a significant decrease in the number of PAC inclusion bodies and increase in the specific PAC activity when *pac* was overexpressed. For the culture of MDΔP7 harboring pTrcKnPAC2902 and pARDegP with IPTG induction at 0.05 mM but without arabinose induction, formation of PAC inclusion bodies was still observed, but there were significantly fewer than in the control experiments with the same induction conditions (Fig. 3). The reduction in the number of PAC inclusion bodies resulted from leaky expression of *degP* by pARDegP, suggesting that a relatively small amount of exogenous DegP would be enough to improve the misfolding of proPAC. The inclusion bodies completely disappeared upon increasing the intracellular DegP concentration for the culture of MDΔP7 harboring pTrcKnPAC2902 and pARDegP with simultaneous IPTG induction at 0.05 mM and arabinose induction at 0.05 g/liter (Fig. 3).

Cell physiology was significantly improved when *degP* was coexpressed. Up to 16% of PAC activity was detected in the extracellular medium for the control experiment, versus only 1% for the culture of MDΔP7 harboring pTrcKnPAC2902 and pARDegP with simultaneous IPTG induction at 0.05 mM and arabinose induction at 0.05 g/liter, suggesting that a much smaller number of cells were lysed when *degP* was coexpressed. As a result, culture performance was significantly improved (Fig. 2). Compared to the control culture of MDΔP7 harboring pTrcKnPAC2902, the final cell density and specific PAC activity were increased by 58% (7.6 versus 4.8 OD_{600} units) and 13% (717 versus 634 U/liter/OD_{600} units), respectively, resulting in an 80% increase in the volumetric PAC activity (5,424 versus 3,011 U/liter). An increase in arabinose induction concentration to 0.1 g/liter did not further improve culture performance (data not shown).

More distinct DegP effect upon extremely high level *pac* expression. The positive effect of DegP on the production of PAC was even more distinct when *pac* was overexpressed with a higher IPTG concentration for induction of 0.1 mM (Fig. 4).

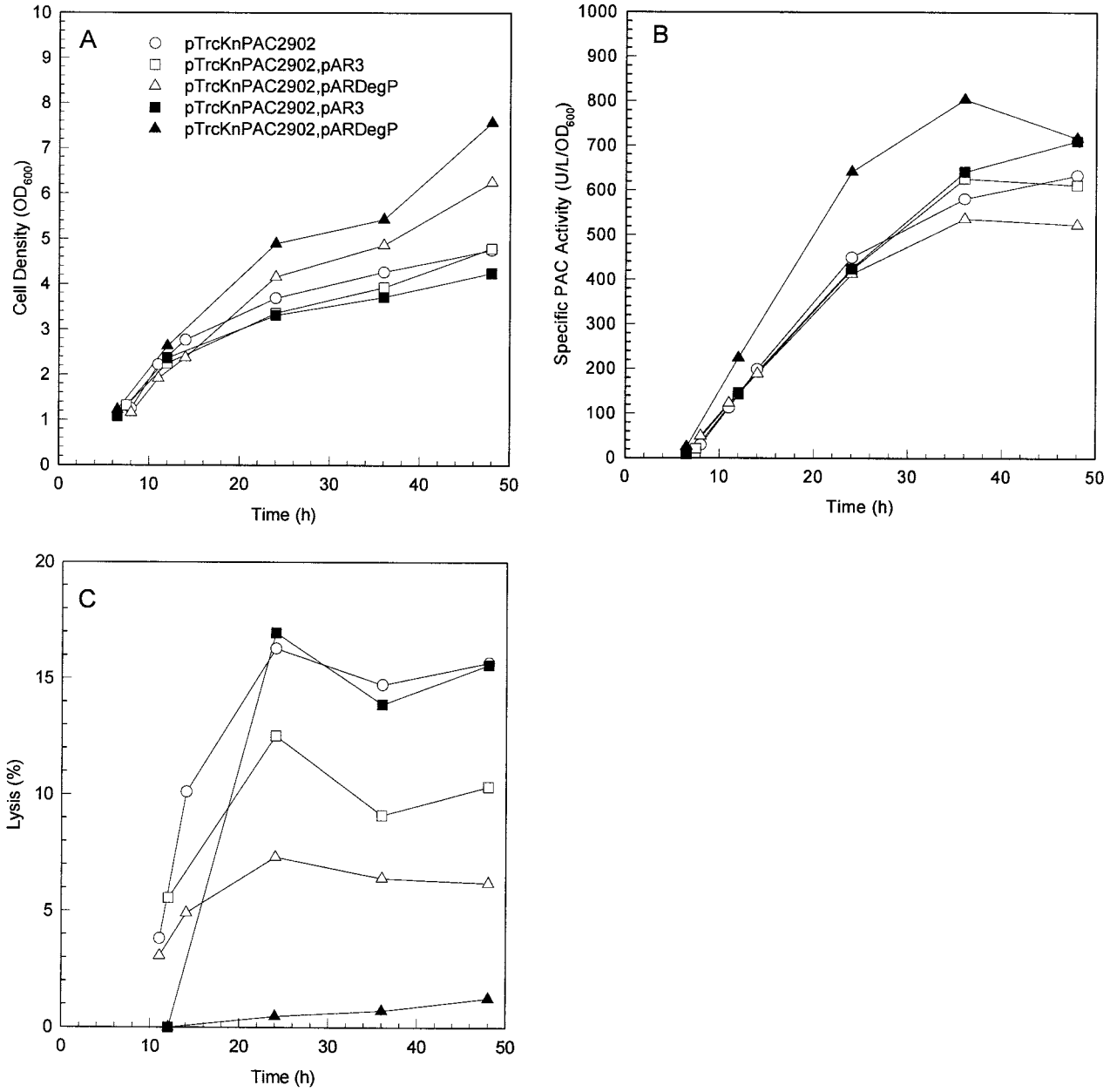


FIG. 2. Effect of DegP on *pac* overexpression: Time profiles of cell density (panel A), specific PAC activity (panel B), and cell lysis level (panel C) for cultures of MDΔP7 harboring various plasmids are shown. The level of cell lysis was defined as the percentage of total PAC activity detected in the extracellular medium. All cultures were supplemented with 0.05 mM IPTG for induction of PAC synthesis when the first sample was taken (i.e., OD_{600} of 1.0 to 1.3). Open symbols represent cultures without arabinose supplementation, whereas solid symbols represent those with arabinose supplementation (0.05 g/liter) for induction of DegP synthesis when the first sample was taken.

With these culture conditions, the number of PAC inclusion bodies was extremely large for the control experiment with MDΔP7 harboring pTrcKnPAC2902 (Fig. 3). The amount of leaky *degP* expression for MDΔP7 harboring pTrcKnPAC2902 and pARDegP was not enough for proper function when arabinose was not added. Cell growth was significantly inhibited due to the extracytoplasmic stress. Increasing the intracellular DegP concentration by adding 0.05 g of arabinose per liter resulted in a significant decrease in the number of PAC inclusion bodies (Fig. 3). Coexpression of *degP* could also improve cell physiology by reducing the level of cell lysis, relieving the inhibition of cell growth. Compared to the control culture of MDΔP7 harboring pTrcKnPAC2902, the final cell density and specific PAC activity were increased by 114% (6.0 versus 2.8 OD_{600} units) and 39% (933 versus 673 U/liter/OD_{600} unit), respectively, resulting in an approximately twofold increase in the volumetric PAC activity (5,593 versus 1,880 U/liter). Up to 50% of PAC activity was detected in the extracellular medium for the control experiment, whereas only 2% was found for the culture of MDΔP7 harboring pTrcKnPAC2902 and pARDegP with simultaneous IPTG induction at 0.1 mM and arabinose induction at 0.05 g/liter.

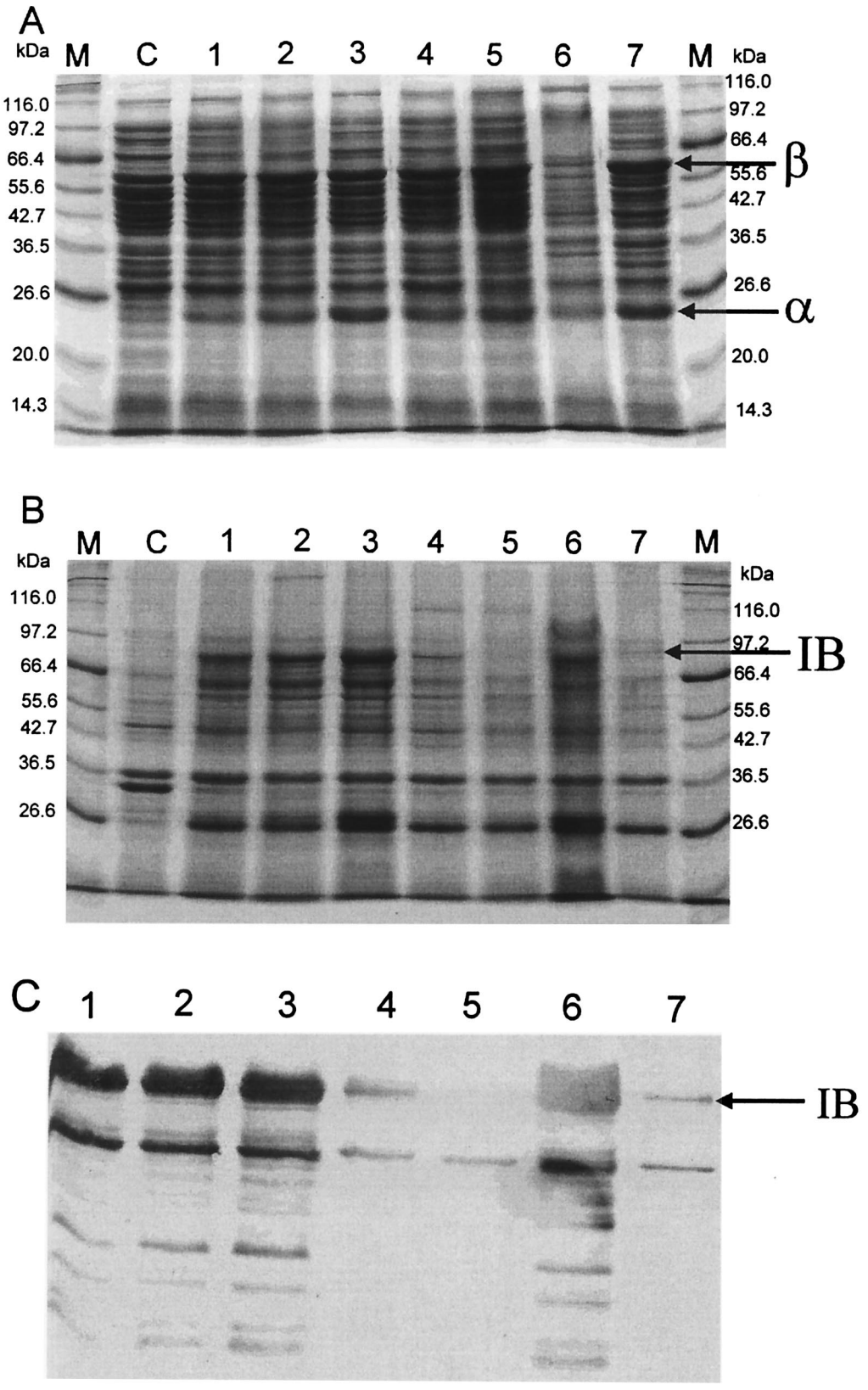


FIG. 3. SDS-PAGE (panels A and B) and immunological (panel C) analyses of protein contents of soluble (panel A) and insoluble (panels B and C) fractions of the last samples of various cultures shown in Fig. 2 and 4. Lanes: M, protein markers; C, pTrcKnPAC2902 without IPTG as the control experiment; 1, pTrcKnPAC2902 with 0.05 mM IPTG; 2, pTrcKnPAC2902 and pAR3 with 0.05 mM IPTG; 3, pTrcKnPAC2902 and pAR3 with 0.05 mM IPTG and 0.05 g of arabinose per liter; 4, pTrcKnPAC2902 and pARDegP with 0.05 mM IPTG only; 5, pTrcKnPAC2902 and pARDegP with 0.05 mM IPTG and 0.05 g of arabinose per liter; 6, pTrcKnPAC2902 and pARDegP with 0.1 mM IPTG only; 7, pTrcKnPAC2902 and pARDegP with 0.1 mM IPTG and 0.05 g of arabinose per liter.

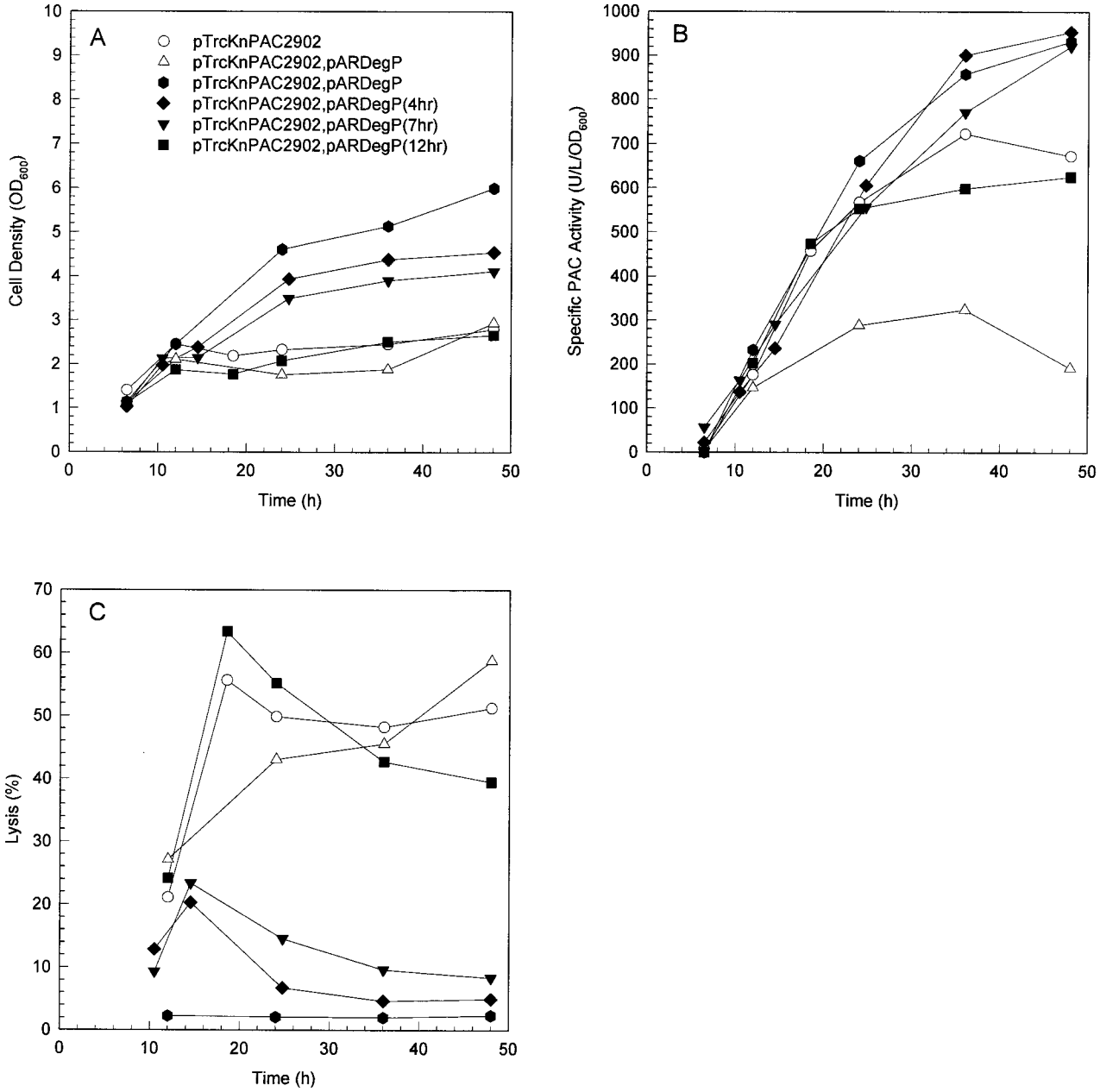
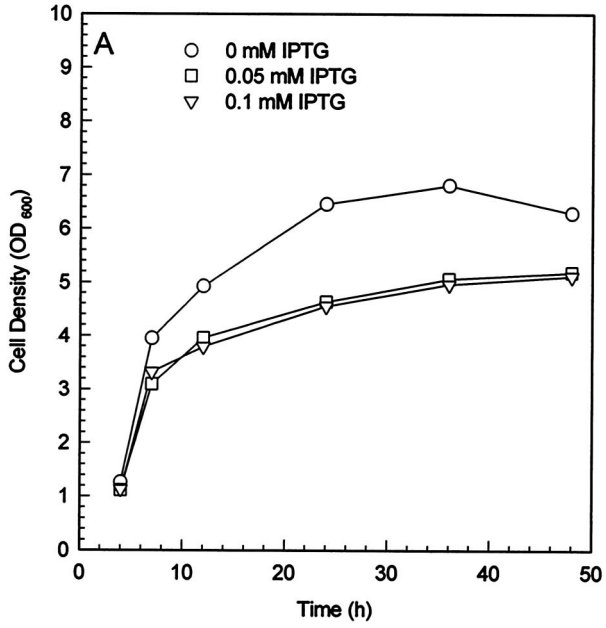
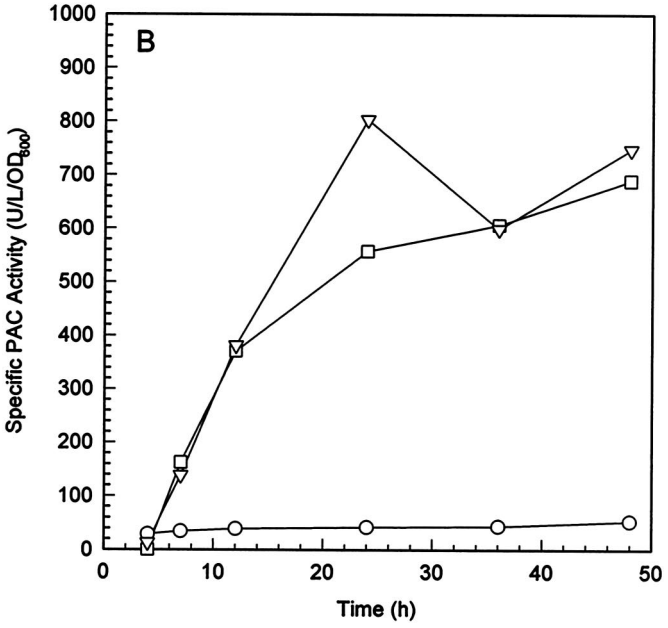


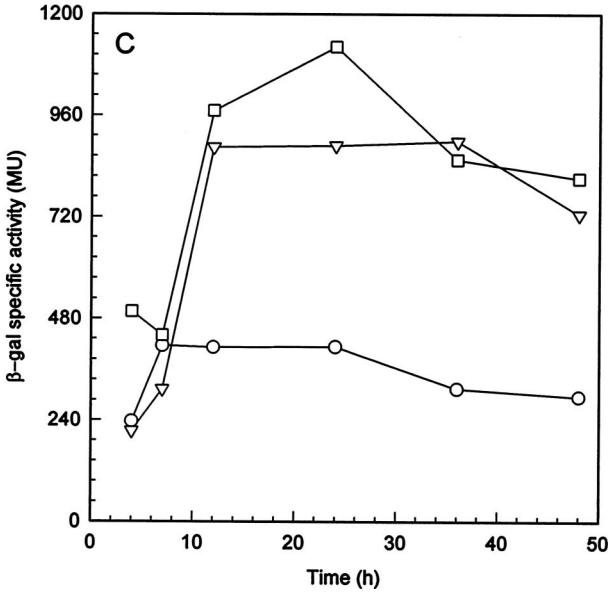
FIG. 4. Effect of DegP on *pac* overexpression. Same as Fig. 2 except all the cultures were supplemented with 0.1 mM IPTG. Solid rhombus, triangle, and square represent cultures supplemented with 0.1 mM IPTG when the first sample was taken but with 0.05 g of arabinose per liter added 4 h, 7 h, and 12 h, respectively, after the first sample was taken.

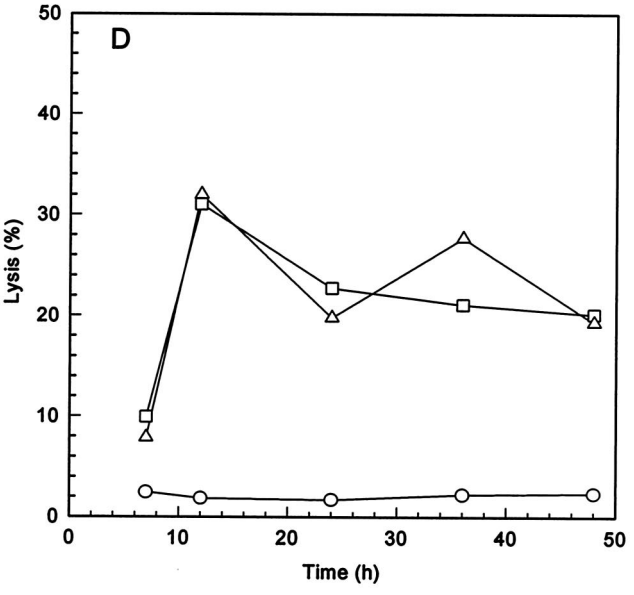
The effect of *degP* induction timing was investigated by cultivations in which *degP* coexpression was induced 4 h, 7 h, and 12 h after *pac* induction (Fig. 4). It appears that the positive effects (i.e., enhanced cell growth, increased *pac* expression, and reduced cell lysis) on *pac* overexpression were not observed until *degP* coexpression. In addition, these effects gradually diminished when *degP* coexpression was delayed and could hardly be observed if *degP* coexpression was delayed by 12 h (Fig. 4). The results suggest that cell physiology improved immediately after *degP* coexpression and that the timing of *degP* coexpression was rather critical. The PAC inclusion bodies formed before *degP* coexpression disappeared gradually toward the end of the cultivations (data not shown). The results suggest that DegP not only prevented further formation of PAC inclusion bodies but also degraded the misfolded proPAC. However, the question of whether the misfolded proPAC was simply degraded or was rescued and subsequently shifted into the productive pathway remains to be answered.

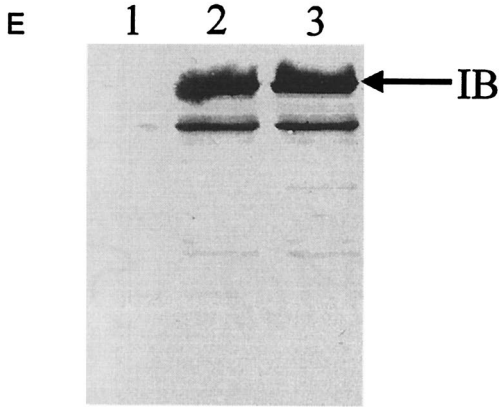
DegP is a heat shock protein induced in response to *pac* overexpression. MC4100λφ[*htrA-lacZ*] contains a single copy of the P_{degP}::*lacZ* transcriptional fusion at the λ phage attachment site of the chromosome of MC4100 (12). The strain can


A
0 mM IPTG
0.05 mM IPTG
0.1 mM IPTG
Cell Density (OD600)
Time (h)


B
Specific PAC Activity (U/L/OD600)
Time (h)


C
β-gal specific activity (MU)
Time (h)


D
Lysis (%)
Time (h)


E
1
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be used to estimate the level of the cell's heat shock response to extracytoplasmic stresses by assaying β-galactosidase activity. With MC4100λφ[*htrA-lacZ*] harboring pTrcKnPAC2902, an increase in *degP* promoter activity was observed as a physiological response to *pac* overexpression, which resulted in slight growth inhibition but significant increases in specific PAC activity, the number of PAC inclusion bodies, and the level of cell lysis (Fig. 5). The results suggest that DegP is a heat shock protein induced in response to *pac* overexpression.

Effect of coexpression of $DegP_{S210A}$ and other DegP-like periplasmic proteases on *pac* overexpression. The role that DegP plays in *pac* overexpression can be identified by comparing culture performance among MDΔP7 harboring pTrcKn PAC2902, MDΔP7 harboring pTrcKnPAC2902 and pARDegP, and MDΔP7 harboring pTrcKnPAC2902 and $pARDegP_{S210A}$. The results are summarized in Fig. 6. Unlike wild-type DegP, $DegP_{S210A}$ could not suppress the physiological toxicity caused by *pac* overexpression. Culture performance (i.e., *pac* expression level, number of PAC inclusion bodies, cell growth, and cell lysis) of MDΔP7 harboring pTrcKnPAC2902 and $pARDegP_{S210A}$ was similar to that of the control experiment with MDΔP7 harboring pTrcKnPAC2902. The results suggest that the protease activity of DegP is responsible for suppression of the physiological toxicity caused by *pac* overexpression (see later discussion).

Since the protease activity of DegP was primarily responsible for suppression of the physiological toxicity caused by *pac* overexpression, it would be interesting to see if any other periplasmic protease had the same phenotype. The effect of DegQ and DegS, two periplasmic serine proteases homologous to DegP, on *pac* overexpression was investigated with MDΔP7 harboring pTrcKnPAC2902 and pARDegQ or pARDegS, and the results are summarized in Fig. 6. Culture performance of MDΔP7 harboring pTrcKnPAC2902 and pARDegQ and of MDΔP7 harboring pTrcKnPAC2902 and pARDegS was similar to or even worse than that in the control experiment with MDΔP7 harboring pTrcKnPAC2902. The results suggest that neither DegQ nor DegS could suppress the physiological toxicity caused by *pac* overexpression.

DISCUSSION

Coexpression of DegP resulted in the following improvements upon *pac* overexpression: (i) it prevented misfolding of proPAC due to a reduction in the number of PAC inclusion bodies; (ii) it shifted PAC synthesis flux from the nonproductive pathway to the productive one due to an increase in *pac* expression level; and (iii) it improved cell physiology due to enhanced cell growth and less cell lysis (Fig. 2, 3, and 4). The result that DegP is a heat shock protein induced in response to *pac* overexpression (Fig. 5) suggests that DegP could suppress the physiological toxicity caused by *pac* overexpression. DegP was also found to be a heat shock protein induced in response to the misfolding of many periplasmic proteins, such as PhoA (13), DsbA′-PhoA (10), MalS (42), OmpF mutants (27), and maltose-binding protein (MalE) variants (3, 28). Misfolding of these proteins is usually the consequence of overexpression of gene products or a folding defect caused by certain mutations. While the heat shock response was initiated primarily for degrading or renaturing misfolded proteins in the periplasm, the increased DegP level might not be high enough for complete function and the misfolded gene products still accumulated as periplasmic inclusion bodies (10, 13, 28). Normally, heat shock proteins express the protease activity for degrading misfolded proteins and/or chaperone activity for renaturing misfolded proteins. We therefore tried to identify the exact role(s) that DegP plays as a heat shock protein in response to *pac* overexpression.

There are at least two hypothetical conditions which are not completely mutually exclusive to explain the positive effect of DegP when *pac* was overexpressed. First, the periplasmic processing machinery was saturated with PAC precursors of proPAC transiently formed in an excess amount. The periplasmic processing of proPAC is initiated by proteolysis on the Thr_{263}-Ser_{264} bond via intramolecular autoproteolysis (15). Two protein chains (i.e., α plus 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 (22, 23). Apparently, the production of PAC was limited by the initiation step, as proPAC was the major aggregation species upon *pac* overexpression. In other words, folding of proPAC in the periplasm was not properly carried out for subsequent autoproteolysis, possibly due to an increased level of the local protein concentration. This limitation possibly can be resolved by periplasmic chaperones. Second, cell physiology was significantly affected as a result of extracytoplasmic stresses, such as protein misfolding and/or failure of degradation of misfolded proteins in the periplasm upon *pac* overexpression. This limitation possibly can be resolved by periplasmic proteases and/or chaperones.

DegP has both protease and chaperone activities (42), by which cell physiology can be improved. For example, DegP was identified as a periplasmic heat shock protein for degrading abnormal proteins in the periplasm when *phoA* was overexpressed (13). Overexpression of *phoA* in a *degP* mutant caused a severe defect in export of several proteins, including PhoA itself, and such a defect was not observed (even in the *degP* mutant) in the absence of *phoA* overexpression (13). The proteolytic activity of DegP could also suppress the physiological toxicity originating from overexpression of a hybrid protein of DsbA′-PhoA in a similar way (10). As a result, DegP was identified as a peripheral membrane protease which degrades misfolded proteins accumulated near the periplasmic side upon extracytoplasmic stresses (13, 30, 40). On the other hand, DegP was also a heat shock protein in response to the misfolding of ovexpressed MalS in the periplasm and the physiological role was, however, proposed to be a chaperone (42).

FIG. 5. Heat shock response to *pac* overexpression. Time profiles of cell density (panel A), specific PAC activity (panel B), specific β-galactosidase activity (panel C), and cell lysis level (panel D) for cultures of MC4100λφ[*htrA-lacZ*] harboring pTrcKnPAC2902 induced with IPTG at 0, 0.05, and 0.1 mM are shown. Immunological analysis of the protein contents of the insoluble fractions of the last samples of various cultures is shown in panel E. Lane 1, no IPTG; 2, 0.05 mM IPTG; 3, 0.1 mM IPTG.

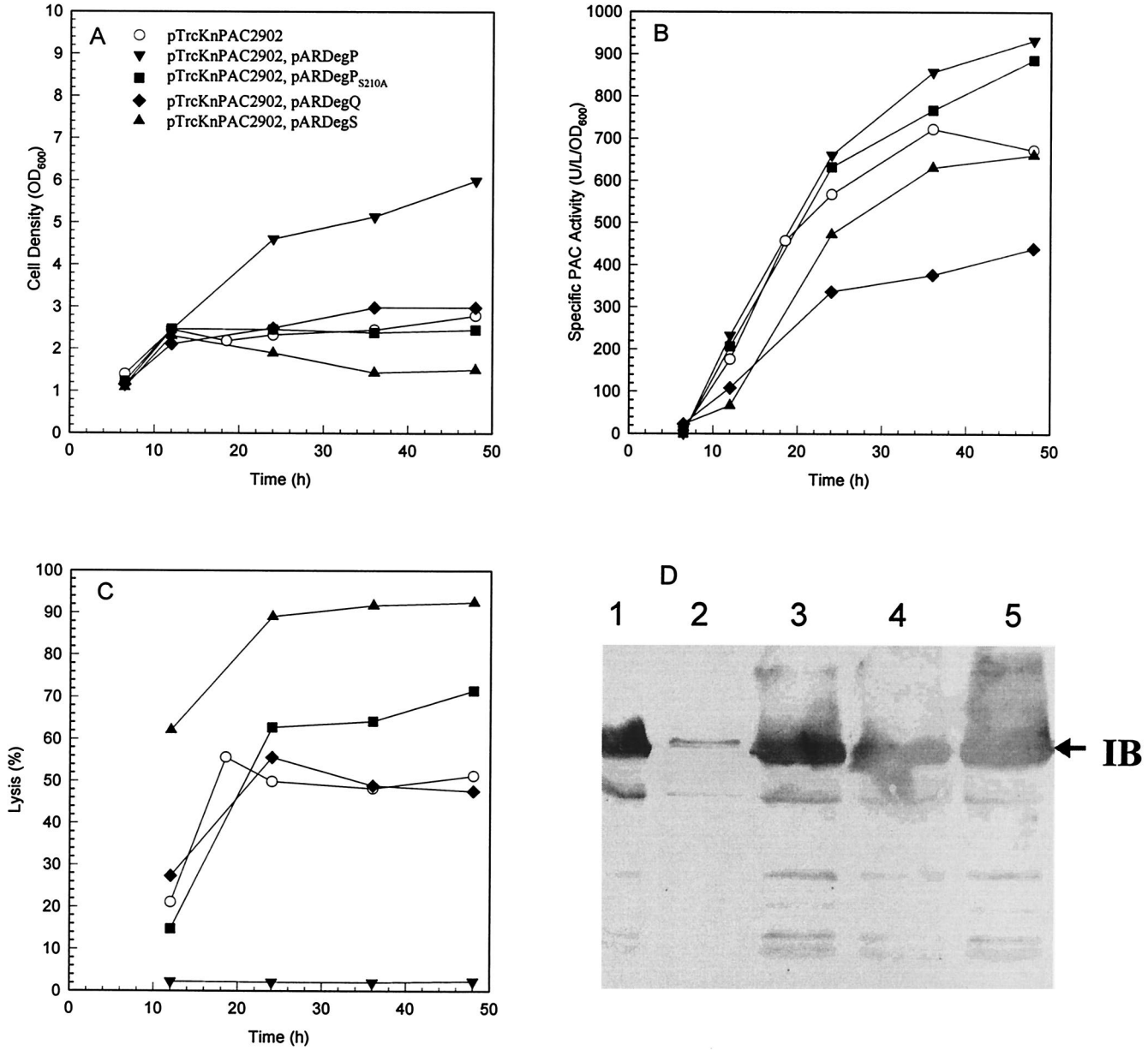

FIG. 6. Effect of various periplasmic proteases on *pac* overexpression. Same as Fig. 2 except the solid symbols represent cultures further supplemented with 0.05 g of arabinose per liter for induction of protease synthesis when the first sample was taken. Immunological analysis of the protein contents of the insoluble fractions of the last samples of various cultures is shown in panel D. Lanes: 1, pTrcKnPAC2902; 2, pTrcKnPAC2902 and pARDegP; 3, pTrcKnPAC2902 and pARDegP$_{S210A}$; 4, pTrcKnPAC2902 and pARDegQ; 5, pTrcKnPAC2902 and pARDegS.

While MalS solubility was improved, the number of MalS inclusion bodies was not reduced, and the in vivo chaperone activity of DegP was not clearly demonstrated (42). It would be interesting to see which activity contributed the improvement documented in Fig. 2 to 4 when *pac* was overexpressed.

DegP is a serine protease whose active center consists of the catalytic triad residues His105, Asp135, and Ser210 (33). The DegP variant harboring the S210A mutation completely lost the protease activity but retained the chaperone activity (41, 42), as the mutation induced minor changes in the tertiary structure of the protein (39). Hence, the role of DegP in *pac* overexpression can be identified with this mutant, DegP$_{S210A}$, being coexpressed. Unlike wild-type DegP, DegP$_{S210A}$ could not suppress the physiological toxicity caused by *pac* overexpression (Fig. 6), suggesting that DegP functioned as a protease to improve culture performance upon *pac* overexpression. Similar approaches were conducted to identify the protease activity of DegP for suppression of the physiological toxicity when the hybrid protein DsbA′-PhoA was overexpressed (10) or the chaperone activity of DegP for suppression of heat shock responses originating from misfolding of MalS (42) and OmpF mutants (27).

More than 10 periplasmic proteases have been identified in *E. coli*. Among them, DegQ and DegS are the two with DegP-like protein function and structure. All three proteins are serine proteases (47). However, only DegP is heat inducible and only *E. coli degP* mutants have a temperature-sensitive phenotype (47). DegQ can functionally substitute for DegP under certain conditions (47). Though DegQ is not a heat shock protein, it can transiently degrade denatured, unfolded proteins which accumulate in the periplasm upon heat shock or extracytoplasmic stresses and/or newly secreted proteins prior to folding and disulfide bond formation (17). Similar to many intracellular proteases, which form large oligomeric complexes, both DegP and DegQ form hexamers or dodecamers in solution (17, 18). DegP and DegQ are similar in size and highly homologous (47). In addition, they both contain two PDZ domains, which are responsible for substrate recognition, and possibly have a common substrate recognition mechanisms (30). On the other hand, DegS is also homologous to DegP, though the homology is not as strong as that of DegQ and DegP (47). It also has the PDZ domain for substrate recognition, but only one (30). The physiological function of DegS was identified to be related to the regulation of σ^E activity (1).

Unlike DegP, neither DegQ nor DegS could suppress the physiological toxicity caused by *pac* overexpression (Fig. 6). The apparent different effects of the two highly homologous proteases, DegP and DegQ, on *pac* overexpression suggest that the improvement in culture performance was not simply caused by DegP protease activity. In that case, the chaperone activity could be another possible improving factor. It is known that DegP can exhibit protease or chaperone activity with temperature as an environmental switch; namely the protease and chaperone activities dominate under high and low temperatures, respectively (42). Hence, the possibility that the positive effect of DegP on *pac* overexpression was caused by the chaperone activity should not be excluded since all the cultivations were conducted at a relatively low temperature of 28°C in this study. The chaperone activity was responsible for shifting the PAC synthesis flux from the nonproductive pathway to the productive one possibly by increasing the solubility of proPAC. In addition, increasing the efficiency of the current limiting step (i.e., autoproteolysis of proPAC) would require a proper folding status of proPAC that could possibly be achieved by DegP chaperone activity.

It should be noted that the chance that DegP protease activity directly assisted the periplasmic processing of proPAC, which consists of several proteolytic steps, would be slim due to the following reasons. First, DegP targets its protein substrates by recognizing a state of protein denaturation rather than specific amino acid sequences in particular proteins (19). The PDZ domains of DegP are responsible for substrate recognition (30). In addition, unfolding of the protein substrates would be essential for their access into the inner chamber of the double ring-shaped DegP, where cleavage of peptide bonds may occur (16). Second, both DegP and DegQ have two PDZ domains and an active center with the catalytic triad residues of His, Asp, and Ser for exhibiting protease activity. One would expect to see the same effect for DegQ as for DegP on *pac* overexpression if DegP protease activity directly assisted autoproteolysis of proPAC.

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