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Stable Expression and Secretion of Polyhydroxybutyrate Depolymerase of *Paucimonas lemoignei* in *Escherichia coli*

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An efficient strategy for the expression and secretion of extracellular polyhydroxybutyrate depolymerase (PhaZ1) of *Paucimonas lemoignei* in *Escherichia coli* was developed by employing the signal peptide of PhaZ1 and a truncated ice nucleation protein anchoring motif (INPNC). Directly synthesized mature form of PhaZ1 was present in the cytoplasm of host cells as inclusion bodies, while a construct containing PhaZ1 and its own *N*-terminal signal peptide (PrePhaZ1) enabled the secretion of active PhaZ1 into the extracellular medium. However, the PrePhaZ1 construct was harmful to the host cell and resulted in atypical growth and instability of the plasmid during the cultivation. In contrast, INPNC-PhaZ1 and INPNC-PrePhaZ1 fusion constructs did not affect growth of host cells. INPNC-PhaZ1 was successfully displayed on the cell surface with its fusion form, but did not retain PhaZ1 activity. In the case of INPNC-PrePhaZ1, the initially synthesized fusion form was separated by precise cleavage of the signal peptide, and active PhaZ1 was consequently released into the culture medium. The amount of PhaZ1 derived from *E. coli* (INPNC-PrePhaZ1) was almost twice as great as that directly expressed from *E. coli* (PrePhaZ1), and was predominantly (approximately 85%) located in the periplasm when cultivated at 22°C but was efficiently secreted into the extracellular medium when cultivated at 37°C.

***Keywords*:** polyhydroxybutyrate depolymerase, signal peptide, ice nucleation protein, recombinant protein, secretion, fusion protein, *Escherichia coli*

Escherichia coli has long been used for the production of recombinant proteins. In this bacterium, some of the expressed recombinant proteins remain in the cytoplasm, whereas others are targeted to the periplasm, outer membrane or extracellular medium. Heterologous proteins targeted to the outer membrane or extracellular medium have several advantages that include facilitation of proper folding due to the oxidizing environment of periplasm, reduction of proteolysis caused by cytoplasmic proteases, and simplified purification (Hannig and Makrides, 1998; Choi and Lee, 2004; Mergulh *et al*., 2005). Therefore, a simple strategy in which recombinant protein is initially synthesized as preprotein bearing a *N*-terminal signal peptide sequence followed by cleavage of the preprotein to produce mature protein during the translocation through the cytoplasmic membrane is widely used for the secretion of recombinant proteins (Shokri *et al*., 2003; Jana and Deb, 2005). However, although a variety of signal sequences have been successfully used, the presence of a signal sequence is not a guarantee of efficient protein translocation, and precise processing into a mature protein does not always occur (Denefle *et al*., 1989; Humphreys *et al*., 2000; Shokri *et al*., 2003). Moreover, expression of such constructs can be toxic, resulting in host cell death, plasmid instability and, consequently, low yields of the desired proteins (Beena *et al*., 2004; Wu and Chang, 2004; Pfeiffer *et al*., 2006).

Recent efforts have focused on overcoming these limitations, since the fusion protein strategy represents a potentially very useful means for the efficient production of heterologous proteins. An attractive candidate fusion partner is ice nucleation protein (INP), an outer membrane protein from ice nucleation active bacteria such as *Pseudomonas syringae* and *Xanthomonas campestris*. INP has been extensively studied and employed to display target proteins on the Gram-negative bacterial surface (see review of Samuelson *et al*., 2002). The expressed INP is stable even in stationary phase cells and does not adversely affect growth of host cells. In particular, the repeating domain in the central region of INP is not essential for the protein to anchor on the outer membrane, so it can be reduced or deleted when used as anchoring motif. To date, proteins successfully expressed in *E. coli* and displayed on the outer membrane using the INP fusion system include various bacterial enzymes (Jung *et al*., 1998a, 1998b, 2003; Yim *et al*., 2006), viral epitopes (Kang *et al*., 2003) and several eukaryotic proteins including single chain antibody (Kwak *et al*., 1999; Bassi *et al*., 2000).

Polyhydroxyalkanoates (PHAs) are a class of naturally occurring polyesters that are synthesized by a wide range of bacteria as a carbon and energy reserve material. PHAs have attracted much commercial attention as promising candidates for potentially useful biodegradable and biocompatible polymers for a wide range of industrial and medical applications (Zinn *et al*., 2001; Kim *et al*., 2007). The most fascinating feature of PHAs is their biodegradability to CO_2 and

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H_2O. The ability to environmentally degrade PHAs depends on the secretion of specific extracellular PHA depolymerases that hydrolyze the polymer to water-soluble products. In addition to a possible application in environmental biotechnology for the eco-friendly management of waste PHAs (Kim and Rhee, 2003), the use of PHA depolymerases for the production of fine chemicals through enzymatic degradation of PHAs may prove to be one of the most important industrial applications of the enzymes (Lee *et al*., 1999; Tokiwa and Calabia, 2004).

The production of microbial PHA depolymerases is normally induced in the presence of PHA. However, PHA depolymerase activity in liquid cultures is typically extremely low, mainly due to the aggregation of sonicated PHA granules in the culture medium. To circumvent this problem, recombinant *E. coli* has been considered as a producer of PHA depolymerase. However, several approaches to produce PHA depolymerases in *E. coli* have so far resulted in poor yields or the formation of inactive inclusion bodies (Jendrossek *et al*., 1995; Jiang *et al*., 2004). Development of a novel strategy for the efficient production of PHA depolymerases in recombinant hosts such as *E. coli* is urgently needed.

Paucimonas lemoignei (formerly *Pseudomonas lemoignei*) is one of the most extensively studied strains capable of producing PHA depolymerases. This organism is an unusual bacterium that possesses at least seven PHA depolymerases (PhaZ1-PhaZ7) (Jendrossek *et al*., 1995; Schober *et al*., 2000; Handrick *et al*., 2001). Among them, the 414 amino acid PhaZ1 is the smallest polyhydroxybutyrate (PHB) depolymerase. As an extracellular enzyme, PhaZ1 has a *N*-terminal signal peptide composed of 37 amino acids that is correctly recognized and cleaved by a leader peptidase at a site between Ala37 and Leu38 (Jendrossek *et al*., 1993). Presently, a novel fusion system for the secretory production of PhaZ1 in *E. coli* was developed by employing the signal peptide of PhaZ1 and ice nucleation protein anchoring motif (INPNC), a truncated INP portion containing *N*- and *C*-terminal domains (Jung *et al.*, 1998b), as a fusion partner. This system proved to be highly effective for stable expression and secretion of soluble PhaZ1 in an active form during the cultivation of host cells.

Materials and Methods

Strains, culture conditions, and plasmids

P. lemoignei (Sei *et al*., 2001) was used as the source of the *phaZ1* gene. It was grown at 30°C in Stinson and Merrick's mineral salts medium (Stinson and Merrick, 1974) supplemented with 50 mM sodium succinate. *E. coli* DH5α and *E. coli* BL21 (DE3) were used as the host for the amplification and conservation of plasmids and the expression of recombinant proteins, respectively. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 0.5% NaCl, pH 7.0). Since complex media such as LB can produce negative effects on the expression of recombinant proteins (Mergulhao *et al*., 2003), the re-

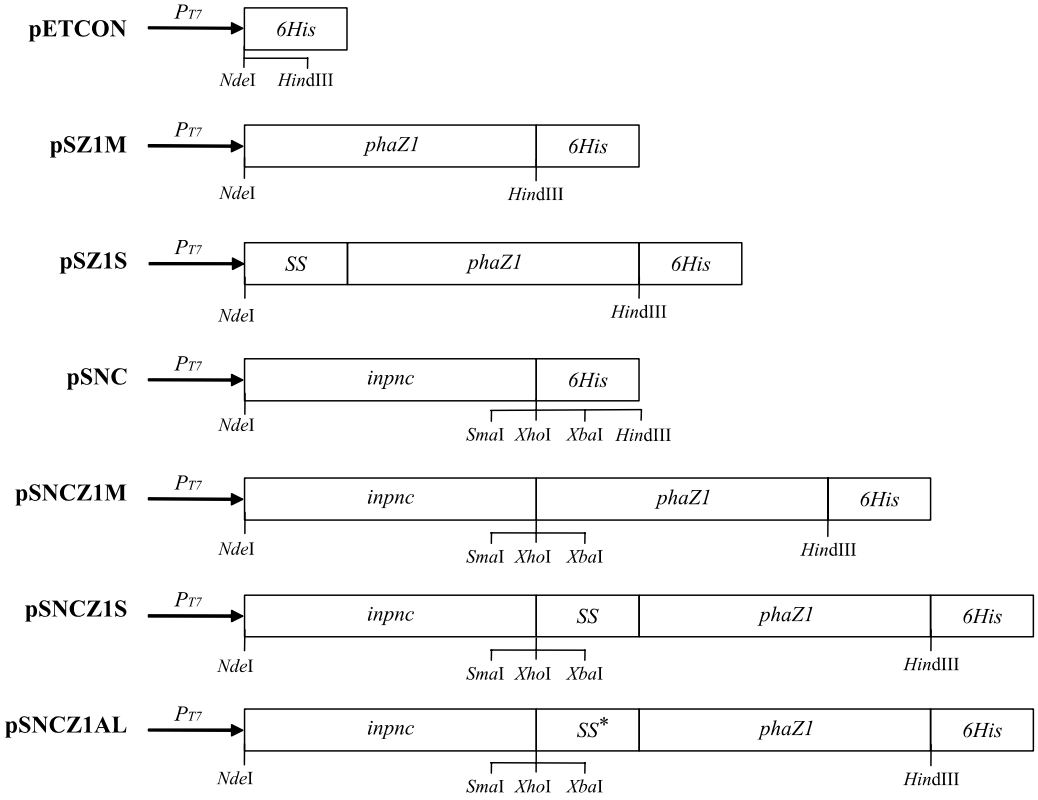

Fig. 1. Structure of expression vectors. All expression vectors were constructed based on pET-20b(+). Abbreviations of P_{T7}, *SS*, and *6His* represent T7 promoter, signal sequence of *pha*Z1 and six histidine coding sequence, respectively. The asterisk in pSNCZ1AL indicates the deleted site of nucleotides coding Ala37 and Leu38.

combinant *E. coli* were cultured in M9 medium supplemented with 0.1% glucose, 0.05% yeast extract, 0.05% tryptone, and 100 μg/ml ampicillin at 22°C or 37°C. Induction of each recombinant protein was facilitated by the addition of 0.5 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) when the optical density at 600 nm (A_{600}) reached 0.4. The plasmid pET-20b(+) (Novagen, USA) composed of the T7 promoter, *N*-terminal *pelB* signal sequence and *C*-terminal His-tag was used to construct expression vectors. The pGEM-T easy vector (Promega, USA) was employed for cloning of polymerase chain reaction (PCR) products. The pJHC11 vector containing the INPNC encoding gene (Jung *et al*., 2003) was used as a PCR template for the amplification of the gene.

Construction of expression vectors

All pET-20b(+)-based expression vectors constructed in this study are shown in Fig. 1. For the construction of the pETCON vector, the *pelB* signal sequence was removed by digestion with *Nco*I and *Eco*RV, followed by ligation after filling the sticky end with a Klenow fragment. The strain carrying only the insert-free pETCON vector was used as a control. The *phaZ1* gene was PCR amplified from the genomic DNA of *P. lemoignei*. A 1251 bp DNA fragment encoding PhaZ1 and its signal sequence was PCR amplified using the primer pairs fw-CATATGCTTGCAAAGCAAATC AAGAAAGCCAATTCAC-3' (containing the *Nde*I reaction site) and bw-AAGCTTGATGGGACTGCAGTTTGCTTTAT CAGTC-3' (primer PhaZ-bw containing the *Hin*dIII reaction site). For amplification of the 1143 bp DNA fragment encoding only PhaZ1, primer fw-CATATGTTGACGCAGGTA TCCAACTTCGGC-3' (containing the *Nde*I reaction site) and primer PhaZ-bw were used. These two PCR products were first cloned into the pGEM-T easy vector and then subcloned into *Nde*I/*Hin*dIII-digested pET-20b(+) to generate pSZ1S and pSZ1M, respectively.

The gene encoding INPNC was PCR amplified from pJHC11 using the primer pairs fw-CATATGACTCTCGAC AAGGCGTTGGTGCTG-3' (containing the *Nde*I reaction site) and bw-TCTAGACTCGAGCCCGGGCTTTACC-3' (containing the *Xba*I reaction site). The resulting PCR product was cleaved with *Nde*I and *Xba*I, and inserted into the same enzyme-digested pET-20b(+) to create the expression vector pSNC. To construct the INPNC-PrePhaZ1 and INPNC-PhaZ1 fusion proteins, each PCR product of 1251 bp and 1143 bp DNA fragment, in which the initiator methionine codon of *phaZ1* was deleted, was cloned using 5'-TCTAGACTTGCAAAGCAAATCAAGAAAGCCAATTC AC-3' (containing the *Xba*I reaction site) and 5'-TCTAGATT GACGCAGGTATCCAACTTCGGC-3' (containing the *Xba*I reaction site), respectively, as an upstream primer and primer PhaZ-bw as a downstream primer. Insertion into the pGEM-T easy vector yielded pGEMZ1S and pGEMZ1M, respectively. After verification of the correct orientation of each PCR product in the pGEM-T easy vector, INPNC gene digested with *Nde*I and *Xba*I was introduced into the same enzyme-digested pGEMZ1S and pGEMZ1M. Finally, each *Nde*I/*Hin*dIII-digested product from pGEMZ1S and pGEMZ1M was introduced to pET-20b(+) to construct pSNCZ1S and pSNCZ1M, respectively. PCR amplification of *phaZ1* with the deletion of Ala37 and Leu38 residues (the recognition site of signal peptidase) was performed using an upstream primer 5'-GAGCTCTTCGTGAGTGTACACGCAGGTAT-3' (containing the *Sac*I reaction site) and primer PhaZ-bw as a downstream primer. This PCR product was inserted into *Sac*I/*Hin*dIII-digested pSNCZ1S to construct the expression vector pSNCZ1AL. All constructed expression vectors were verified by DNA sequencing.

Subcellular fractionation of *Escherichia coli*

During the cultivation of recombinant strains in the modified M9 broth, 2 ml samples were collected after 6 h and 12 h induction. One milliliter was centrifuged and the supernatant was directly used to measure the activity of PhaZ1 located in the extracellular medium. The insoluble cells were osmotically shocked as recommended by pET system manual (Novagen) to prepare periplasmic-located enzyme. For 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses (see below), these fractionated samples were concentrated five-fold using an equal volume of 20% trichloroacetic acid. The remaining 1 ml of culture was centrifuged, washed twice with phosphate buffered saline (PBS) and the final pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.9). The cells in the suspension were broken by sonic oscillation twice for 2 min. Insoluble material was removed by centrifugation (12,000×g for 10 min) and the supernatant fraction was used for the determination of enzyme in whole cell lysates. All experiments were carried out on ice or at 4°C.

SDS-PAGE and Western blot analysis

SDS-PAGE was carried out on a 10% gel and proteins were visualized with Coomassie Brilliant Blue R-250. For immunodetection, separated proteins were transferred (1 h at 100 mA) from a gel onto a polyvinylidene difluoride membrane. The membrane was washed twice with Tris Buffered Saline (TBS)-Tween buffer (20 mM Tris-HCl; pH 7.5, 500 mM NaCl, and 0.05% Tween 20) before incubating for 1 h in blocking buffer consisting of 3% bovine serum albumin in TBS-Tween buffer at room temperature. The blocked membrane was reacted with anti-INPNC-antibody kindly provided by Dr. Heung-Chae Jung, KRIBB, Korea, or anti-Penta·His-antibody (QIAGEN, USA) as the primary antibody. Enhanced chemiluminescence detection (Amersham, USA) was carried out using horseradish peroxidase conjugated with anti-rabbit-lgG (Sigma-Aldrich, USA) as the secondary antibody.

Immunofluorescence microscopy

For the preparation of rabbit anti-PhaZ1 antibody, PhaZ1 purified from the periplasmic fraction of *E. coli* BL21 (pSNCZ1S) using a Ni^{2+} affinity column (QIAGEN) was injected into a rabbit with two booster injections over the next 1.5 months. Anti-PhaZ1 serum was obtained from the rabbit blood. To examine whether the INPNC and fusion proteins were expressed on the surface of the transformed cells, transformants were grown at 22°C for 4 h, then 0.5 mM IPTG was added and incubated for an additional 3 h with slow shaking. The harvested cells were resuspended in PBS with 1% skim milk containing a 1:50 dilution of either rabbit anti-INPNC antibody or rabbit anti-PhaZ1-antibody, and incubated at room temperature with mild inversion.

After recovery by centrifugation, the primary antibody-cell complex was washed five times with PBS. For immunofluorescence microscopy, a 1:100 dilution of anti-rabbit IgG-fluorescein isothiocyanate (Sigma-Aldrich, USA) was added and incubated in the same conditions. After incubation, the cells were washed five times with PBS and examined using an Olympus BX51 fluorescent microscope (Olympus, Japan).

Preparation of PHB granules and latex suspension

PHB was produced from *Ralstonia eutropha* KHB-8862 as described previously (Chung *et al*., 2001) and PHB granules were isolated from the cells as described previously (Kim *et al*., 2002). Latex suspension of the granules was accomplished by ultrasonic dispersion in distilled water.

Assay for PHB depolymerase activity

PHB depolymerase activity was detected on modified M9 agar containing 0.3% (w/v) PHB granules. After incubation at 37°C for 20 h, enzyme activities were confirmed by the formation of a clear zone around each colony. Enzyme activity of the PHB suspension was also determined by measuring the turbidity decrease at 650 nm. The 2 ml assay mixture (final OD_{650} 1.5) consisting of 0.1 ml of the PHB suspension; 0.1 ml of each enzyme solution prepared from the extracellular medium, periplasmic space or sonicated whole cell and 1.8 ml of 100 mM Tris-HCl (pH 7.9) was incubated at 37°C for 1 h. One unit of enzyme was defined as the decrease of the value of OD_{650} by 1 absorbance unit per h.

Plasmid stability

Plasmid stability within cells during flask culture was determined by diluting the culture samples in PBS, plating onto LB-agar and incubating at 22°C or 37°C for 16 h. One hundred colonies were replica-plated onto LB agar and LB-agar containing 100 μg/ml ampicillin, prior to incubation for 24 h. Plasmid stability was defined as the percentage of ampicillin-resistant colonies.

Results

Construction of expression vectors

We first constructed the PhaZ1 expression vectors, pSZ1M and pSZ1S, for direct synthesis of only the mature form (PhaZ1) and of the premature form containing the *N*-terminal signal peptide (PrePhaZ1), respectively. After transformation into *E. coli* BL21 (DE3), each transformant was cultured on LB agar containing 100 μg/ml ampicillin at 37°C for 16 h. The transformants differed markedly in their colony sizes. *E. coli* BL21 (pSZ1S) formed very tiny colonies, which were even smaller than one-fifth of the BL21 (pSZ1M) and BL21 (pETCON) colonies (data not shown) suggesting an inhibitory effect of the pSZ1S construct on host cell growth. However, on modified M9 agar containing PHB granules, only BL21 (pSZ1S) produced a visible clear zone around the colonies (Fig. 2), which indicated that PrePhaZ1 was functionally expressed and also could be secreted into the extracellular medium. In contrast, the mature PhaZ1 synthesized in BL21 (pSZ1M) was not secreted, but instead accumulated in the cytoplasm as inclusion bodies (data not shown).

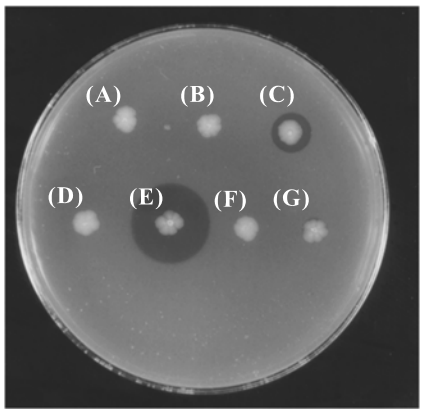

Fig. 2. Activity test of PHB depolymerase (PhaZ1). Testing was done on modified M9 agar plate containing 0.3% (w/v) PHB granule, 100 μg/ml ampicillin and 0.5 mM IPTG. *E. coli* BL21 harboring pETCON (A), pSZ1M (B), pSZ1S (C), pSNC (D), pSNCZ1S (E), pSNCZ1M (F), and pSNCZ1AL (G) were grown at 37°C for 20 h. The enzyme activities were confirmed by formation of a clear zone around the colonies.

The second approach involved display of the PhaZ1 target protein on the cell surface using INPNC as a fusion partner. Two fusion vectors were constructed: pSNCZ1S for the expression of INPNC-PrePhaZ1 and pSNCZ1M for the expression of INPNC-PhaZ1. Unlike pSZ1S, which negatively affected growth of the host cells, both pSNCZ1S and pSNCZ1M produced transformants that formed average sized colonies on LB agar. However, BL21 (pSNCZ1M) did not produce any clear zone on the modified, PHB granule containing M9 agar, whereas BL21 (pSNCZ1S) produced a clear zone around the colony that were much larger than those evident with BL21 (pSZ1S) (Fig. 2).

Analysis of expressed proteins by SDS-PAGE and Western blotting

Considering the role of INPNC as an anchoring motif for

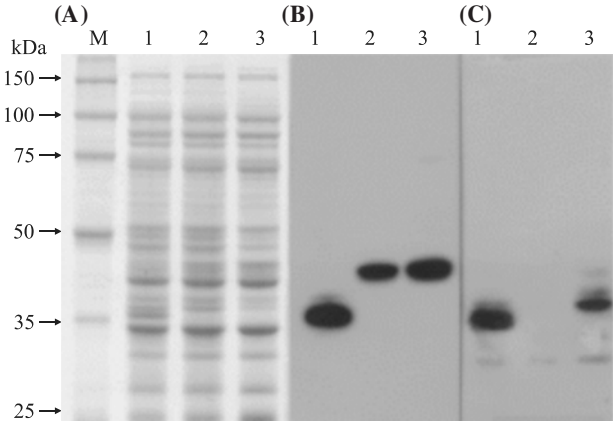

Fig. 3. Analysis of recombinant proteins expressed in *E. coli* BL21 cells harboring pSNC (lane 1), pSZ1S (lane 2), pSNCZ1S (lane 3) by 10% SDS-PAGE (A) and Western blot with anti-Penta His-antibody (B) and anti-INPNC antibody (C). Lane M, molecular mass markers (kDa).

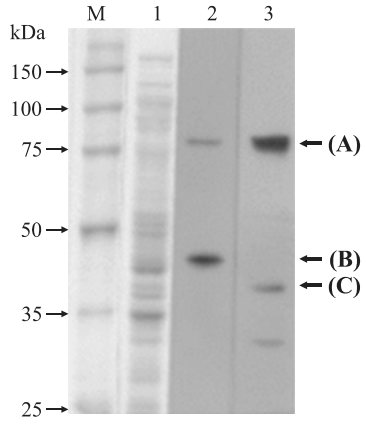

Fig. 4. Analysis of recombinant proteins expressed in *E. coli* BL21 harboring pSNCZ1AL by SDS-PAGE (lane 1) and Western blotting with anti-Penta His-antibody (lane 2) and anti-INPNC antibody (lane 3). Arrows indicate the fusion form of INPNC-PrePhaZ1 (A), PhaZ1 (B), and INPNC-Pre (C).

display of target protein on host cell surfaces, we sought to determine why INPNC-PrePhaZ1 produced the markedly larger halo exemplified in Fig. 2, and the precise nature of the signal peptide located in the middle of the fusion protein during translocation. To solve these questions, it was necessary to identify whether the fusion protein was correctly synthesized and maintained its fusion form after translocation. SDS-PAGE and Western blotting analyses were performed with whole cell lysates of the BL21 (pSNC), BL21 (pSZ1S) and BL21 (pSNCZ1S) transformants (Fig. 3). All constructions contained six His residues tagged at the C-terminus of each recombinant protein, which could be easily detected with anti-Penta·His-antibody. INPNC (M*r,* 36 kDa) expressed from BL21 (pSNC) and PhaZ1 (M*r,* 44 kDa) expressed from BL21 (pSZ1S) were detected by immunoblotting with anti-Penta·His-antibody (Fig. 3B). Interestingly, however, an INPNC-PrePhaZ1 fusion protein band with a predicted M*r* of 84 kDa that was anticipated to have been expressed in BL21 (pSNCZ1S) was not evident. Instead, a prominent PhaZ1 band was mainly detected. In the Western blotting performed with anti-INPNC-antibody, the INPNC band from BL21 (pSNCZ1S) was up-shifted (Fig. 3C, lane 3), perhaps due to the presence of the *C*-terminal signal peptide. These results substantiated a novel phenomenon that the signal peptide located between INPNC and PhaZ1 could be functionally processed in a similar fashion as the *N*-terminally located signal peptide, to liberate mature PhaZ1 from the fusion protein.

For further confirmation that the signal peptide from INPNC-PrePhaZ1 was precisely processed and consequently liberated the authentic mature protein, we constructed the pSNCZ1SDAL vector, in which the signal peptidase recognition site (Ala37 and Leu38) was deleted, for the expression of the fusion protein. Results from Western blotting showed that the efficiency of cleavage event was greatly reduced and most of the expressed protein retained its fusion form (84 kDa) (Fig. 4). In this experiment, bands that corresponded to PhaZ1 and INPNC-Pre were also faintly detected, which might be attributed to inept non-specific cleavage near the deleted recognition site.

To accurately identify the cleavage site, *N*-terminal amino acid sequence analyses of PhaZ1 samples purified from BL21 (pSZ1S) and BL21 (pSNCZ1S) were undertaken. The results revealed the identical sequence (LTQVSNFGTN) in both enzymes. Collectively, the results clearly demonstrate that the PhaZ1 signal peptide located either in the *N*-terminus or in the middle of the fusion protein was functionally recognized by the signal peptidase in *E. coli* and consequently contributed to the liberation of the mature PhaZ1.

Localization of fusion proteins on the surface of *E. coli*

To investigate whether the INPNC-PhaZ1 and INPNC-Pre fusion proteins synthesized by BL21 (pSNCZ1M) and BL21 (pSNCZ1S), respectively, were correctly located on the *E.*

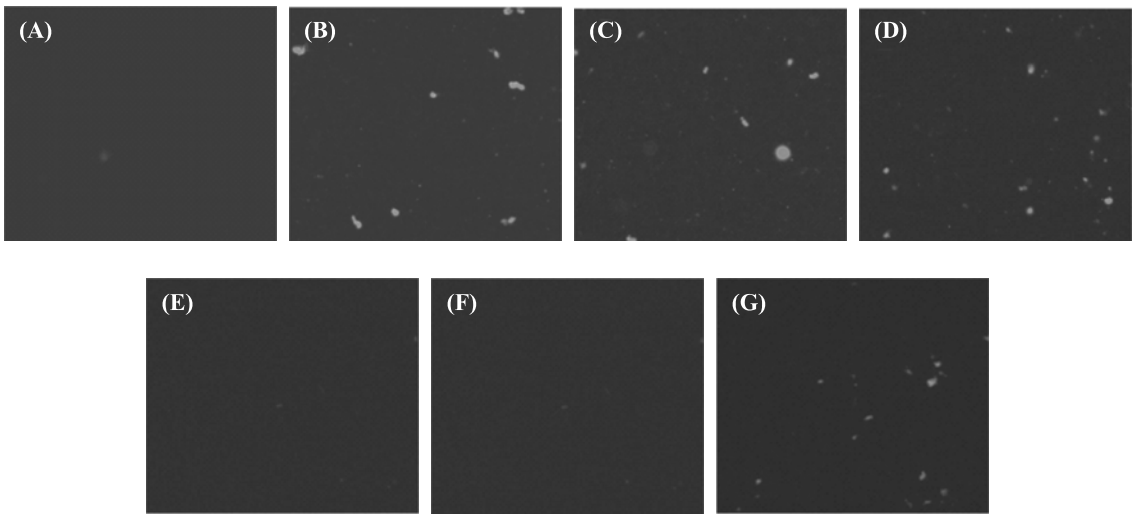

Fig. 5. Immunofluorescence micrographs of *E. coli* BL21 cells harboring pETCON (A), pSNC (B and E), pSNCZ1S (C and F), and pSNCZ1M (D and G). The cells were probed with anti-INPNC antibody (A-D) and anti-PhaZ1 antibody (E-G).

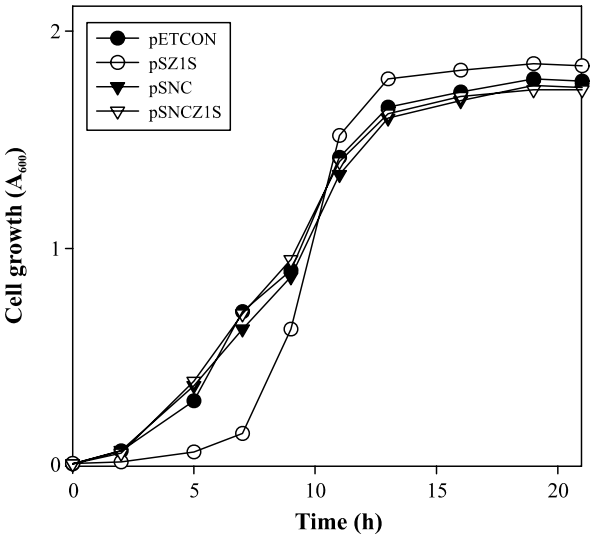

Fig. 6. Growth of *E. coli* BL21 cells harboring pETCON, pSZ1S, pSNC, and pSNCZ1S in modified M9 medium at 37°C. The induction of each recombinant protein was performed by the addition of 0.5 mM IPTG at 0.4 A_{600}.

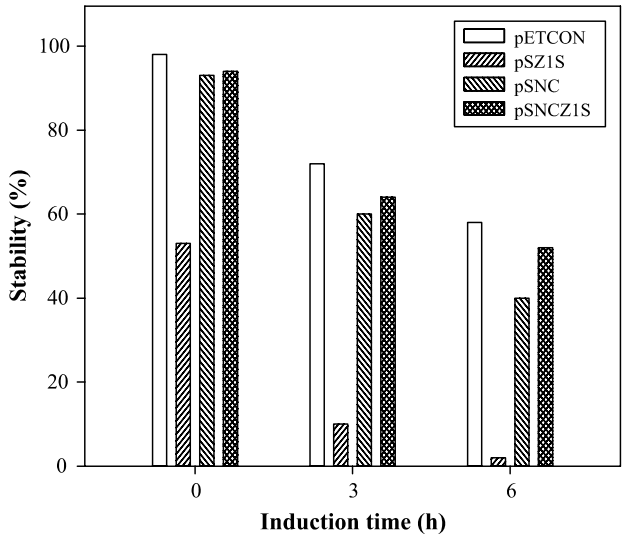

Fig. 7. Plasmid stability test of *E. coli* BL21 harboring pETCON, pSZ1S, pSNC and pSNCZ1S. During cultivation at 37°C (A) and 22°C (B), samples were analyzed before induction and after 3 h and 6 h induction. The numbers of colonies were counted from LB-Amp agar media after growth at 37°C for 16 h.

coli surface, immunofluorescence microscopy was carried out using rabbit anti-INPNC-antibody or rabbit anti-PhaZ1-antibody as the primary antibody (Fig. 5). Similar to BL21 (pSNC) cells, BL21 (pSNCZ1S), and BL21 (pSNCZ1M) cells were efficiently stained by fluorescein isothiocyanate-labeled secondary antibody against rabbit anti-INPNC-antibody. On the other hand, only BL21 (pSNCZ1M) cells expressing INPNC-PhaZ1 were evident by immunofluorescence microscopy using rabbit anti-PhaZ1-antibody. These results are consistent with the surface anchorage of both INPNC-PhaZ1 and INPNC-Pre (derived from INPNC-PrePhaZ1 after cleavage of PhaZ1).

Differential effects of PrePhaZ1 and INPNC-PrePhaZ1 on the host cells

Although BL21 (pSZ1S), in common with BL21 (pSNCZ1S), could secrete active PhaZ1, the poor growth of BL21 (pSZ1S) on LB agar suggested that expression of PrePhaZ1, even at a low level under non-inducing conditions, might be harmful to the growth of host cells. To clarify the effects of each recombinant protein on the growth of *E. coli* and to estimate the relative yields of PhaZ1 produced during the cultivation, each *E. coli* BL21 transformant harboring pETCON, pSNC, pSZ1S or pSNCZ1S was cultivated in the modified M9 medium. Based on an assumption that temperature may influence the location of recombinant proteins (Jana and Deb, 2005), all transformants were incubated both at 22°C and 37°C. As shown in Fig. 6, BL21 (pSNC) and BL21 (pSNCZ1S) grew equally as well as the control strain at 37°C, while BL21 (pSZ1S) grew poorly initially and began to overgrow after 7 h cultivation. Similar findings were noted during cultivation at 22°C (data not shown).

The plasmid stability of above four constructs was also monitored. As shown in Fig. 7, BL21 (pSZ1S) easily lost the plasmid during cultivation and most of the cells (>80%) did not carry the plasmid after 3 h induction, whereas the pSNC and pSNCZ1S constructs were as stable as the control vector. The results in Fig. 6 and 7 indicate that the PrePhaZ1 construct (pSZ1S) was unstable in the host cell and negatively affected growth, which consequently resulted in the lower production of the PHB depolymerase compared to the INPNC-PrePhaZ1 (pSNCZ1S) construct.

Table 1 shows the localization of PhaZ1 from BL21 (pSZ1S) and BL21 (pSNCZ1S) cells grown at 22°C and 37°C. Measurement of PHB depolymerase activities in fractionated samples revealed that total activity of PhaZ1 from

Table 1. Activity and localization of PHB depolymerase (PhaZ1) expressed in *E. coli* BL21 cells harboring pSZ1S and pSNCZ1S

	Activity (U/ml) after 6 h induction				Activity (U/ml) after 12 h induction			
	22°C		37°C		22°C		37°C	
	pSZ1S	pSNCZ1S	pSZ1S	pSNCZ1S	pSZ1S	pSNCZ1S	pSZ1S	pSNCZ1S
Whole cell	1.38	4.96	0.99	2.36	1.06	4.81	0.90	2.12
Periplasm	1.21	4.86	0.85	2.17	1.01	4.72	0.76	1.93
Medium	1.50	0.70	1.80	3.47	1.94	0.94	2.40	3.92

BL21 (pSNCZ1S) was almost two-fold higher than that from BL21 (pSZ1S), regardless of growth temperature. PhaZ1 from BL21 (pSNCZ1S) was mostly located in the periplasmic space (85%) at 22°C, but was more predominant in the extracellular medium (60%) at 37°C. In contrast, PhaZ1 from BL21 (pSZ1S) was mostly located in the extracellular medium (52%∼65%) and its localization was not apparently influenced by growth temperature.

Discussion

The aim of our study was to develop a vector system useful for the expression and secretion of PHA depolymerase in *E. coli* using PhaZ1 as the model enzyme. Even though extracellular secretion of recombinant proteins in *E. coli* has been successfully performed using various *N*-terminal signal sequences, not all synthesized target proteins with this system produce satisfactory results. In particular, some recombinant proteins are toxic to the host cells or affect host cell physiology when they are expressed (Shokri *et al*., 2003). Previous attempts to express PHA depolymerase genes in *E. coli* have been unsuccessful for unknown reasons (Jendrossek *et al*., 1995; Jiang *et al*., 2004). To circumvent this problem, *Bacillus subtilis*, a Gram-positive bacterium that lacks an outer membrane has been considered as a suitable host for the production of PHB depolymerase (Braaz *et al*., 2002).

In the present work, the directly synthesized mature PhaZ1 was insoluble and thus, remained in the cytoplasm of host cell as inclusion bodies. Although the secretion of active PhaZ1 could be achieved from the secretory format containing its own *N*-terminal signal sequence (PrePhaZ1), the expression of this construct was toxic to the host. In contrast, the fusion forms INPNC-PrePhaZ1 and INPNC-PhaZ1 were synthesized without damaging the host cell. Particularly, it was found that the fusion system of INPNC and signal peptide of PhaZ1 (INPNC-PrePhaZ1) has unique structural and functional features that are useful for the efficient production of PhaZ1. Firstly, the signal peptide located next to INPNC (36 kDa) operates during translocation, liberating mature PhaZ1. Secondly, the fusion partner appears to enhance solubility, obviating PhaZ1 inclusion body formation in the cytoplasm. Thirdly, INPNC abolishes the toxic effect of PhaZ1 expression observed using the PrePhaZ1 construct, possibly via the modulation of PhaZ1-mediated toxicity in the cytoplasm before cleavage of the precursor region. This possibility also applies to the fusion form of INPNC-PhaZ1, which was soluble and was displayed on the outer membrane, even though it did not retain the enzyme activity probably due to incompatible secondary and tertiary structures of PhaZ1 (Samuelson *et al*., 2002).

Most of the PhaZ1 derived from INPNC-PrePhaZ1 was presently located in the periplasmic space at 22°C but in the extracellular medium at 37°C. This temperature-dependent localization might be due to increased membrane permeability at the high temperature that permits easier translocation of PhaZ1 from the periplasm to the extracellular medium. A similar phenomenon was also found in the expression of PrePhaZ1, but the portion of extracellularly-secreted enzyme was greater regardless of growth temperature. During the cultivation of BL21 (pSZ1S), we observed the formation of bubbles in the liquid growth medium and detected many cytoplasmic proteins from the SDS-PAGE analysis for cell-free culture fluid of BL21 (pSZ1S) (data not shown). Concerning the BL21 (pSZ1S) cells, it is conceivable that the increased enzyme content in the extracellular medium might be attributed to the toxic effect of PrePhaZ1 construct on the cells, and the resultant partial cell lysis during cultivation.

In conclusion, the present results suggest that the novel fusion system, INPNC-PrePhaZ1, is useful for the expression of recombinant PhaZ1 that is toxic to the host cell. By employing this fusion system, it is expected that a target protein can be guided out of the cytoplasm by the action of INPNC and automatically liberated at least into the periplasmic space with the aid of signal sequence. Further experiments will focus on the application of this fusion system for the production of other heterologous proteins.

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