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Enhancement of Excretory Production of an Exoglucanase from *Escherichia coli* with Phage Shock Protein A (PspA) Overexpression

Wang, Y. Y.[1], Z. B. Fu[1,2], K. L. Ng[1], C. C. Lam[1], A. K. N. Chan[1], K. F. Sze[1], and W. K. R. Wong[1]*

[1]*Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China*
[2]*GlaxoSmithKline, Microbial and Cell Culture Development, 709 Swedeland Rd., King of Prussia, PA 19406, USA*

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Production of recombinant proteins by excretory expression has many advantages over intracellular expression in *Escherichia coli*. Hyperexpression of a secretory exoglucanase, Exg, of *Cellulomonas fimi* was previously shown to saturate the SecYEG pathway and result in dramatic cell death of *E. coli*. In this study, we demonstrated that overexpression of the PspA in the JM101(pM1VegGcexL-pspA) strain enhanced excretion of Exg to 1.65 U/ml using shake-flask cultivation, which was 80% higher than the highest yield previously obtained from the optimized JM101(pM1VegGcexL) strain. A much higher excreted Exg activity of 4.5 U/ml was further achieved with high cell density cultivation using rich media. Furthermore, we showed that the PspA overexpression strain enjoyed an elevated critical value (CV), which was defined as the largest quotient between the intracellular unprocessed precursor and its secreted mature counterpart that was still tolerable by the host cells prior to the onset of cell death, improving from the previously determined CV of 20/80 to the currently achieved CV of 45/55 for Exg. The results suggested that the PspA overexpression strain might tolerate a higher level of precursor Exg making use of the SecYEG pathway for secretion. The reduced lethal effect might be attributable to the overexpressed PspA, which was postulated to be able to reduce membrane depolarization and damage. Our findings introduce a novel strategy of the combined application of metabolic engineering and construct optimization to the attainment of the best possible *E. coli* producers for secretory/excretory production of recombinant proteins, using Exg as the model protein.

Keywords: *Escherichia coli*, Excretory production, Exoglucanase, Membrane potential, Phage shock protein A

*Corresponding author
Phone: +852-2358-7299; Fax: +852-2358-1552;
E-mail: bcwkrw@ust.hk

As a robust microbial workhorse, *Escherichia coli* has been employed to produce many pharmaceutically or biotechnologically valuable proteins such as growth hormones, interferons, interleukins, tumor necrosis factors, and plasminogen activators [3, 19]. Secretory or extracellular (excretory) production of heterologous proteins by *E. coli* has many advantages over intracellular synthesis, including simplified downstream processing, enhanced biological activity, higher product stability and solubility, and N-terminal authenticity of the expressed protein. In the biopharmaceutical industry, purification of the excreted protein without going through cell lysis is relatively simple and largely reduces the possibility of endotoxin contamination, thus lowering the production cost and enhancing the product quality. In the biofuel industry, excretory production of recombinant cellulases is a must, since hydrolysis of the insoluble cellulosic biomass inevitably takes place in the extracellular milieu.

Although excretory expression of heterologous proteins in *E. coli* is increasingly preferred compared with intracellular production, the transport of proteins into the culture medium often results in low production yield and poor cell growth [21, 22]. Previously, we reported that saturated translocation of Exg, an exoglucanase encoded by the *cex* gene of *C. fimi*, in the SecYEG channel would cause severe cell death, thus resulting in serious yield loss of excreted Exg [4, 11]. The detrimental effects were attributable to the limited capacity of the SecYEG channel, which is the most commonly employed secretion pathway in *E. coli* [4, 13]. A threshold ratio of pre-protein to mature protein (Pre/Mat RQ) in the cell lysate, designated the critical value (CV), was shown to be an important index to help achieve efficient protein secretion as well as high cell viability in *E. coli* [5]. The CV of Exg has been determined to be 20/80 [5], reflecting the maximum level of the Pre/Mat RQ of Exg tolerable to the host cell. Once the Pre/Mat RQ level exceeds its CV, severe cell death will be triggered [5]. Therefore, a sublethal

level of the heterologous protein acceptable to the host cell is preferred to enable the maintenance of high cell density as well as high-level protein production per cell. Previously, we demonstrated that by fine-tuning the expression level of Exg in *E. coli*, the maximum secretion capacity of the host cell was derived [5]. Moreover, we established a facile and reliable protocol of viable cell counts to predict the maximum tolerable level of a secretory protein, thereby enabling fast access to a secretion level that was close to the CV [20]. According to these findings, we have developed efficient *E. coli* systems capable of expressing the highest levels of excreted Exg [5] and excreted Eng, an endoglucanase encoded by the *cenA* gene of *C. fimi* [20], reported in the literature so far.

Expression of the *E. coli* phage shock protein A (PspA), a 25.3 kDa peripheral membrane protein, is induced under membrane stress conditions [8, 14]. SecYEG or TAT pathway blockage might result in a marked expression of PspA [7, 14]. Oligomers of PspA were shown to bind to membrane phospholipids and suppress proton leakage, thereby helping maintain the membrane potential [9, 10]. In this study, we report that high levels of secretory expression of Exg could inhibit endogenous PspA expression. Protein translocation through the SecYEG channel requires ATP and proton motive force (PMF) [15], and the lethal effect triggered by excessive protein secretion [13] was thus speculated to be attributable to insufficient supply of PspA to maintain the energetic state of the membrane under stress conditions. Regardless of the DNA construct employed, PspA expression was elevated, and the higher levels of PspA were shown to be capable of enhancing excretory production of Exg in *E. coli*. Concomitantly, the elevated levels of PspA were shown to be able to maintain membrane polarization and integrity, despite efficient secretory expression of Exg detected in the same host. These findings helped formulate a strategy of combined application of metabolic engineering and construct optimization in developing potentially the most capable recombinant *E. coli* producers, for the excretion of the maximum possible level of a desired heterologous protein.

Materials and Methods

Chemical Reagents

Propidium iodide (PI), *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4$_{(3)}$), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and L-arabinose were from Sigma (St. Louis, MO, USA). Other chemical reagents were also obtained from Sigma, unless otherwise specified.

Escherichia coli Strain, DNA Manipulation, and Restriction and Modifying Enzymes

E. coli JM101 [(*lac-proAB*), *supE*, *thi1*; F', *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15, *traD36*] [21] was the host for expression and propagation of plasmids. Polymerase chain reaction (PCR) and overlap extension PCR were performed as previously described [22] with the High Fidelity KOD HiFi DNA Polymerase (Novagen, Madison, WI, USA). Other DNA techniques were performed according to standard protocols [16]. Restriction and modifying enzymes were purchased from Pharmacia (Uppsala, Sweden), Promega (Madison, WI, USA), and New England Biolabs (Beverly, MA, USA). The reaction buffers were provided by the suppliers and used as instructed. BigDye sequencing kits were from Applied Biosystems, USA.

Plasmid Construction

The 669 bp *pspA* gene (GenBank Accession No. 945887) encoding the *E. coli* phage shock protein PspA was amplified by PCR using the *E. coli* JM101 genomic DNA as the template and primers 1 and 2 (Table 1). After cleavage with *Eco*RI and *Xba*I, the PCR product was inserted into plasmid pBAD22 restricted by the same two enzymes right downstream from the *araB* promoter to obtain the intermediate plasmid, pBAD22-pspA, in which *pspA* expression was controlled by the *araB* promoter and terminated by the *rrnB* terminator. The 2.5 kb BAD cassette fragment containing the *araC* gene together with its own promoter, and the *pspA* gene fused next to the *araB* promoter, was then amplified by employing plasmid pBAD22-pspA as the template and primers 4 and 5 (Table 1). The PCR product was restricted with *Kpn*I and cloned into plasmids

Table 1. Primers used in this study.

Primer No.	Sequence 5'–3'
1	CGAATTCCATGGGTATTTTTCTCGC
2	GCTCTAGAGCTTATTGATTGTCTTGCTTC
3	GCTCTAGAGGGGTACCTTAATGATGGT G ATGATGGTGTTGATTGTCTTGCTTC
4	GTGGTACCATCGATGCATAATGTGC
5	GCGGTACCAGAGTTTGTAGAAACGC
6	ACGAGCCATCAATGCCTGCTGGCG
7	GCATTGATGGCTCGTTTCGAATCTTTC

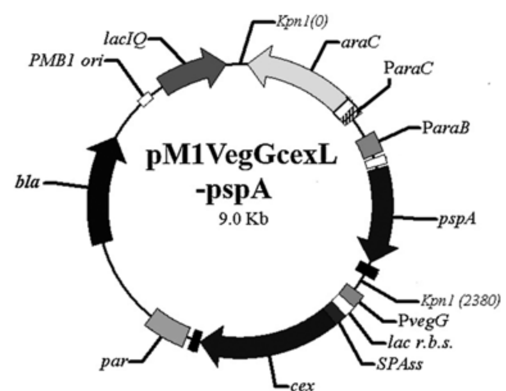

Fig. 1. Schematic representation of the pM1VegGcexL-pspA plasmid. Components shown in the plasmid include *cex*, coding sequence for mature Exg; *pspA*, coding sequence for *E. coli* phage shock protein A (PspA); *bla*, beta-lactamase gene conferring resistance to ampicillin; *lac*IQ, *lac*I^q gene overexpressing the Lac repressor; *par*, the partition locus of the plasmid pSC101; P*araC, araC* promoter; P*araB, araB* promoter; P*vegG, vegG* promoter; *lac r.b.s., lac* operator and ribosome binding site; and *SPAss,* staphylococcal protein A leader sequence. Arrows indicate directions of gene expression.

ptacIQpar8cex [11] and pM1VegGcexL [5] restricted by *Kpn*I to get the final constructs, ptacIQpar8cex-pspA and pM1VegGcexL-pspA (Fig. 1), respectively. As a result, the *cex* gene (encoding Exg of *C. fimi*) [11] in the ptacIQpar8cex-pspA and pM1VegGcexL-pspA constructs was expressed under the control of an IPTG-inducible *tac* promoter and the *vegG* promoter [20], respectively, whereas the *pspA* gene was expressed under the control of an arabinose-inducible *araB* promoter. To generate a 75 bp deletion in the *pspA* gene, two fragments were first amplified using the primer pairs 4 and 6, and 5 and 7 (Table 1), and pM1VegGcexL-pspA as the template. The two fragments were then employed to generate a 2.4 kb fusion product by overlap extension PCR using primers 4 and 5. The fusion product was restricted with *Kpn*I, and cloned into plasmid pM1VegGcexL restricted by *Kpn*I to obtain the final construct, pM1VegGcexL-pspAΔ_{25}.

Culture Conditions

E. coli transformants were grown at 30°C in 2× YT medium (Tryptone 16 g/l, yeast extract 10 g/l, NaCl 5 g/l) supplemented with 70 µg/ml of ampicillin (Amp). For solid media, bacto agar was added at a concentration of 1.5% (w/v). In a time-course study, a 250 ml flask containing 50 ml of growth medium was inoculated with a freshly grown colony and cultured in a shaker run at 200 rpm and 30°C until the A_{550} value reached 0.5. The culture was divided into two halves, each of them supplemented with a final concentration of 0.1 mM of IPTG, and one of them also with 4 mM L-arabinose. Growth was allowed to continue, and samples were then withdrawn at specified intervals from both cultures for viable cell counts, Exg activity assay, and flow cytometry. Viable cell counts on agar plates and Exg activity assay were repeated 3 times with samples retrieved from three individual experiments. For high cell density shake-flask cultivation, cell cultures were grown at 30°C and 400 rpm in a modified terrific broth (MTB) [16], which contained, per liter, 12 g tryptone, 24 g yeast extract, 2.3 g KH_2PO_4, 12.5 g K_2HPO_4, 0.4% (w/v) glucose, 1 g $MgSO_4 \cdot 7H_2O$, 3 ml trace metal solution, and 140 mg Amp. The trace metal solution contained, per liter, 0.162 g $FeCl_3 \cdot 6H_2O$, 0.0144 g $ZnCl_2 \cdot 4H_2O$, 0.12 g $CoCl_2 \cdot 6H_2O$, 0.012 g $Na_2MoO_4 \cdot 2H_2O$, 0.006 g $CaCl_2 \cdot 2H_2O$, 1.9 g $CuSO_4 \cdot 5H_2O$, 0.5 g H_3BO_3, and 37% (v/v) HCl.

Analytical Methods

Cell density was measured spectrophotometrically at A_{550}. Cell supernatants were prepared from culture samples by centrifugation at 12,000 ×*g* for 5 min. The activity of Exg (*p*NPCase) in hydrolyzing *p*-nitrophenyl-cellobioside (*p*NPC) was assayed as described previously [11]. One unit (U) of *p*NPCase produces 1 µmol of *p*-nitrophenol per minute. To obtain cell lysate for Exg activity assay, the pellet of 1 ml cell culture was resuspended in 120 µl of Tris-Cl buffer (50 mM, pH 8.0), followed by an addition of 83 µl of EDTA solution (0.25 M, pH 8.0). The cell mixture was incubated on ice for 5 min, followed by treatment with 120 µl of lysozyme solution (10 mg/ml) at 37°C for 20 min. After the addition of 83 µl of lysis buffer (10 mM EDTA, 10% Triton X-100, and 50 mM Tris.Cl, pH 8.0), the tube was inverted gently to obtain cell lysate.

Protein Preparation and Analysis by SDS–PAGE and Western Blotting

Whole cell lysate was prepared as previously described [5] by resuspending the cell pellet (from 1 ml culture prepared at 4°C using a benchtop centrifuge) in 0.2 ml of 1× lysis buffer [20 mM Tris-Acetate, pH 7.0; 1% (w/v) SDS], followed by thorough mixing and boiling the lysate for 5 min. The protein samples were resolved on a 10% (w/v) Tris-Tricine polyacrylamide separating gel as described by Schägger and von Jagow [17], and electrophoretically transferred onto a polyvinylidine fluoride (PVDF) membrane, which was then developed with an anti-Exg or anti-PspA serum. The results were quantified using the ImageJ software (NIH, USA) [1]. Goat anti-rabbit antibody conjugated with horseradish peroxidase and the ECL Western detection system were from Amersham Bioscience, UK. Anti-PspA antibody was a gift from Prof. Jan Tommassen (Utrecht University, The Netherlands).

Flow Cytometric Analysis

Flow cytometric assays were carried out using a Beckman XL flow-cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The optical filters were set up to measure propidium iodide (PI) fluorescence at 630 nm and *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC4_{(3)}$) fluorescence at 525 nm [6, 12]. Signal logarithmic amplifiers were used in all detectors. The flow rate of a sample was kept at 100–200 events per second. Total collected events were 20,000 per sample.

Samples taken from different intervals of a time-course study were diluted to 10^6 cells/ml with freshly prepared Dulbecco's buffered saline (DBS) [supplemented with 0.05% (v/v) Tween 20, 4 mM EDTA, and 0.1% (w/v) Tryptone, pH 7.2]. The diluted cultures were then stained with either PI or $DiBAC4_{(3)}$ fluorescent dye for 10 min in the dark. The working concentrations of PI and $DiBAC4_{(3)}$ in DBS were 5 µg/ml and 10 µg/ml, respectively. Healthy cells collected in the early exponential growth phase were stained with PI or $DiBAC4_{(3)}$ to serve as negative controls. Cells thermally treated at 70°C for 10 min served as positive controls for membrane damage. Cells decoupled by 10 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were employed as positive controls for membrane depolarization.

Results

Effects of Hyperexpression of Secretory Exg on the Expression of Endogenous PspA

To investigate whether the expression of endogenous PspA, which is involved in numerous membrane stresses, might be affected by the hyperexpression of secretory Exg, JM101 (ptacIQpar8cex) was employed for time-course studies. The induced culture showed dramatic cell death within 2 hr, whereas the non-induced culture showed normal growth [4]. The endogenous PspA expression in both the induced and non-induced cultures was quantified using an anti-PspA antibody as described in Materials and Methods. Although the level of PspA kept on increasing within the first 2 hr in the non-induced culture (Fig. 2A), no significant increase in PspA expression was detected in the induced culture. The amount of PspA at the 2 hr time point in the non-induced culture was 2.6-fold higher than that in the induced counterpart (Fig. 2B). The results support the conclusion that hyperexpression of Exg imposes an inhibitory effect on the expression of endogenous PspA.

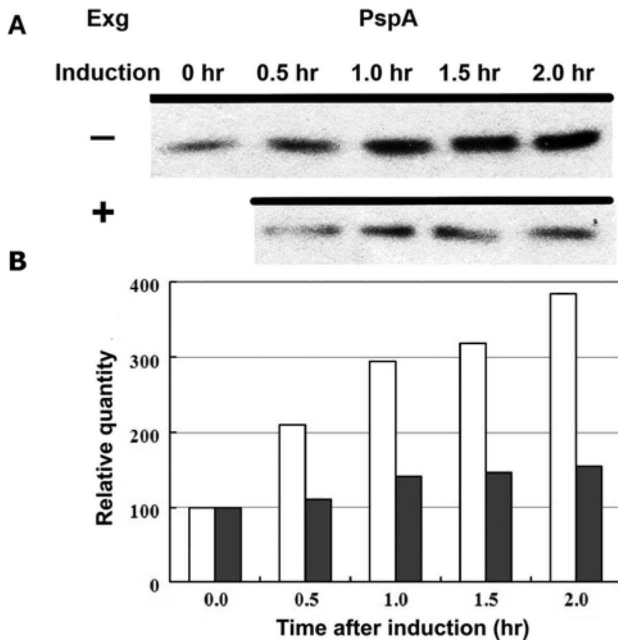

Fig. 2. Effects of hyperexpression of secretory Exg on endogenous PspA expression.

JM101 (ptacIQpar8cex) cultures were grown in 2× YT as described in Materials and Methods. Cultures were then treated with (induced) or without IPTG. Cell lysates were prepared from different culture volumes to obtain 5×10^6 cells at each time point. **A**. Western blot analysis of JM101 (ptacIQpar8cex) cultures with an anti-PspA antibody. **B**. PspA was quantified by optical scanning as described in Materials and Methods. White columns represent measurements of the non-induced samples; gray columns represent measurements of the induced samples.

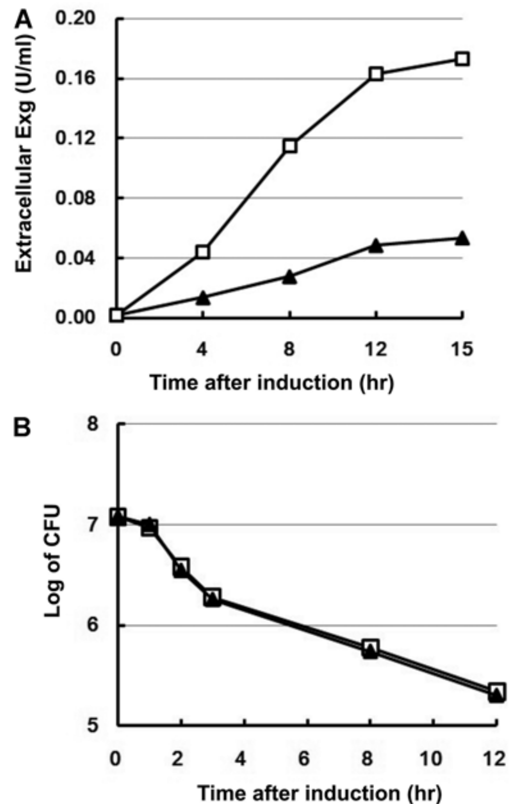

Fig. 3. Effects of PspA overexpression on excretory production of Exg in JM101 (ptacIQpar8cex-pspA).

JM101 (ptacIQpar8cex-pspA) cultures were grown in 2× YT as described in Materials and Methods. Cultures were then induced with IPTG and arabinose or with only IPTG. **A**. Excreted Exg activities. **B**. Viable cell counts of the cultures induced with IPTG and arabinose or with only IPTG. The figures show the cultures induced with IPTG and arabinose (−□−) or with only IPTG (−▲−). CFU: colony forming units.

Enhancement of Excretory Expression of Exg by PspA Overexpression

Previously, JM101 (ptacIQpar8cex) [4, 11] and JM101 (pM1VegGcexL) [5] were employed for extracellular production of Exg under the control of the *tac* promoter and the *vegG* promoter, respectively. To examine whether overexpression of PspA might benefit the secretory and hence excretory expression of Exg, JM101 (ptacIQpar8cex-pspA) and JM101 (pM1VegGcexL-pspA) (Fig. 1) were employed for time-course studies. The additional *pspA* gene in the two plasmids was under the control of the arabinose-inducible *araB* promoter. Overexpression of PspA was triggered by the addition of arabinose, whereas excretory expression of Exg was induced with IPTG. Although the JM101 (ptacIQpar8cex-pspA) culture induced with both arabinose and IPTG produced higher levels of extracellular Exg than its counterpart induced with only IPTG, the overall yield of excreted Exg from either culture was low (Fig. 3A). The low yields of excreted Exg were attributable to the dramatic cell death of the two cultures (Fig. 3B), which expressed a high, but lethal, level of Exg upon IPTG induction of the *tac* promoter [4, 11] contained in the ptacIQpar8cex-pspA construct.

When JM101 (pM1VegGcexL-pspA) was grown and induced with IPTG and arabinose, the culture showed high viable cell counts, which remained high even at the later points of the study (ca. 10^8 cells/ml) (Fig. 4A). The cell viability of the culture was equally high when it was induced with only IPTG (Fig. 4B). However, the excreted Exg from the culture induced with both IPTG and arabinose reached a high level of 1.65 U/ml at the end point (24th hr), which was 80% better than its counterpart induced with only IPTG (0.9 U/ml at the end point) (Fig. 4C). When the total cell-associated Exg activity in the cell lysate was determined, the sample collected at the end point of the culture induced with both IPTG and arabinose gave rise to a remarkably high Exg activity of 2.67 U/ml, which was over 100% higher than the culture induced with only IPTG (Fig. 4D). These results showed that PspA overexpression enhanced the production of both total Exg and excreted Exg activities in JM101 (pM1VegGcexL-pspA).

To examine whether PspA overexpression might increase the production of Exg in JM101 (pM1VegGcexL-pspA) grown to high cell densities, the transformant was grown in rich MTB medium and shaken at 400 rpm to facilitate

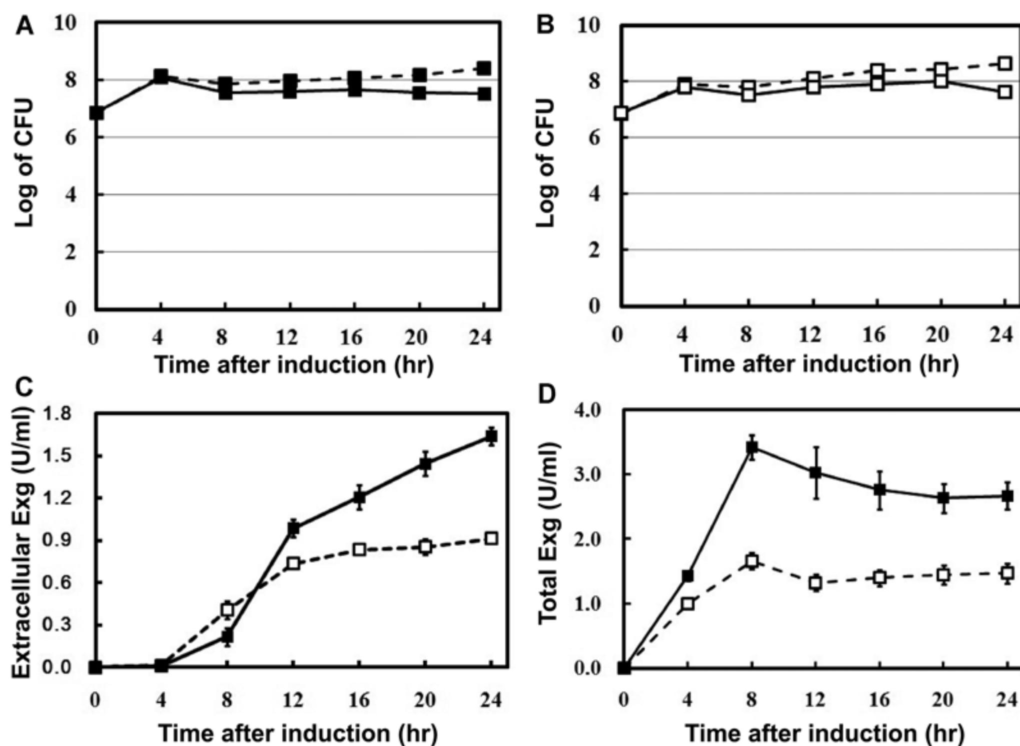

Fig. 4. Effects of PspA overexpression on the production of Exg in JM101 (pM1VegGcexL-pspA).
JM101 (pM1VegGcexL-pspA) cultures were grown in 2× YT as described in Materials and Methods. Cultures were then induced with IPTG and arabinose or with only IPTG. **A**, **B**. Viable cell counts of the cultures induced with IPTG and arabinose (■) and with only IPTG (□), respectively. The cell counts enumerated on plain (dashed lines) and Amp-supplemented (solid lines) agar plates are shown. CFU: colony forming units. **C**. Excreted Exg activities. **D**. Total cell-associated Exg activities. The figures show the activities of the culture induced with IPTG and arabinose (–■–) or induced with only IPTG (--□--).

better oxygen transfer. The highest viable cell counts reached 10^{10} CFU/ml upon induction with both IPTG and arabinose. The excreted Exg determined at the end point (24th hr) of the cultivation was 4.5 U/ml, which was 2.7-fold higher than that achieved with 2× YT medium (data not shown), supporting the enhanced application of PspA overexpression in high cell density fermentation.

No Improvement of Excretory Expression of Exg with Overexpression of a Truncated PspA

To examine whether enhanced excretory expression of Exg was due to the overexpressed PspA, plasmid pM1VegGcexL-pspAΔ25, in which a short sequence encoding a 25 amino acid segment forming a helix-coil-helix-turn-helix motif in the secondary structure of PspA (which was presumably essential for the bioactivity of PspA) was deleted as described in Materials and Methods, was constructed to express a defective PspA. The truncated PspA was confirmed by Western blotting with the anti-PspA antibody to possess a reduced size of around 21 kDa (data not shown). When growth of its *E. coli* JM101 transformant was induced with both IPTG and arabinose, the excreted Exg detected at the end point (24th hr) was 0.51 U/ml, which was 40% lower than that of its counterpart induced with only IPTG. No significant difference in cell viability was observed in both cultures, in which the viable cell counts remained high (ca. 10^8 cells/ml) throughout the cultivation (data not shown). The results support the conclusion that overexpression of PspA would result in enhanced secretory expression of Exg.

Correlation of PspA Overexpression with the Critical Value of Exg

To investigate the effect of PspA overexpression on the conversion of Pre-Exg to Mat-Exg, lysate samples collected from JM101 (pM1VegGcexL-pspA) cultures induced with both IPTG and arabinose, or with only IPTG, were analyzed. Both the excreted and total Exg activities of the former culture were shown to be notably higher, 80% and 130%, respectively, than those of the latter one at some points of their growth (Fig. 4). The overexpressed PspA and the processing of Pre-Exg to Mat-Exg were detected with anti-PspA and anti-Exg antibodies, respectively (Fig. 5A). The Pre-Exg to Mat-Exg relative quantities (Pre/Mat RQ) increased to 45/55 at the 16 h point after induction of the culture with IPTG and arabinose (Fig. 5C); the ratio remained the same until the end point (24th hr) was reached (Fig. 5C). The Pre/Mat RQ value also increased, but to a

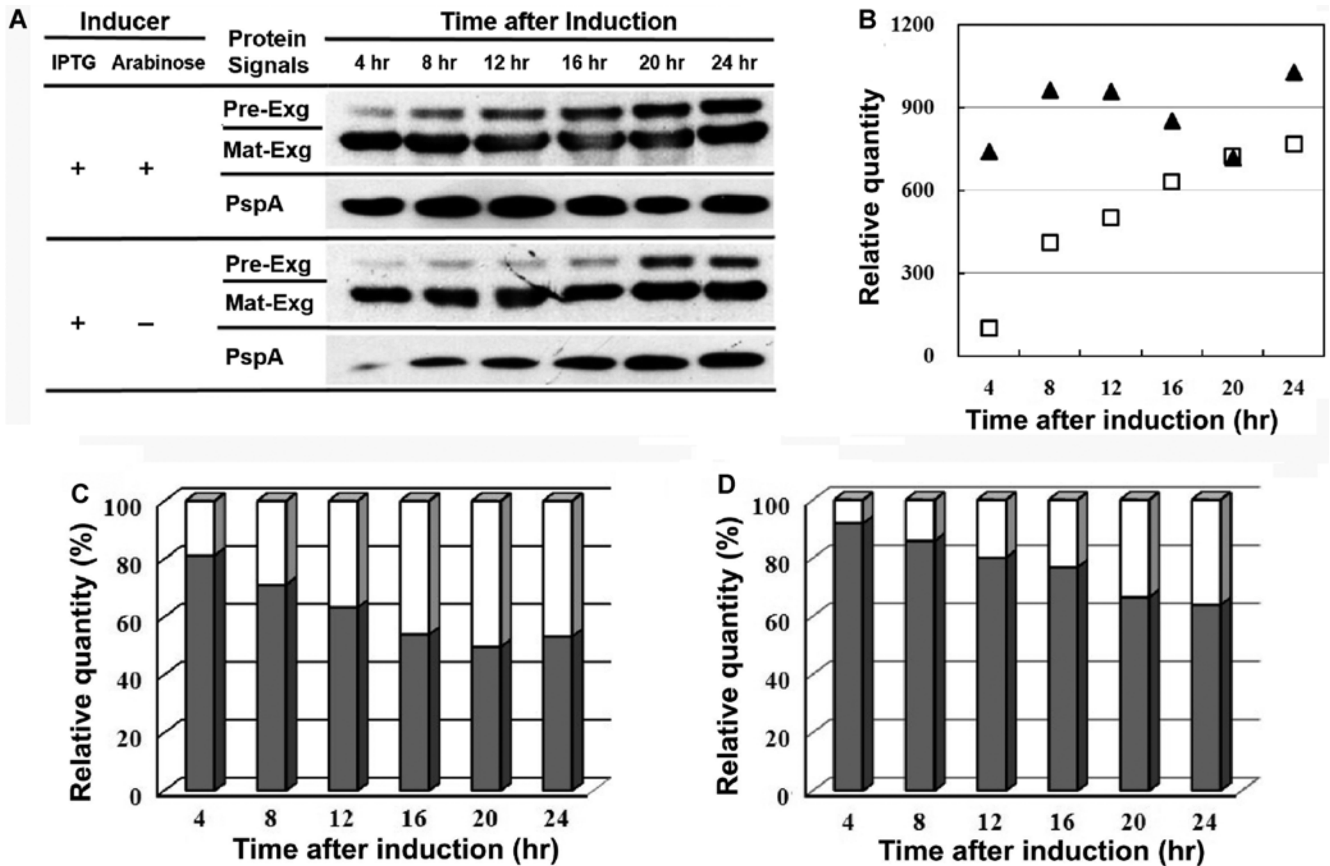

Fig. 5. Effects of PspA overexpression on the conversion of Pre-Exg to Mat-Exg.
JM101 (pM1VegGcexL-pspA) cultures were grown in 2× YT as described in Materials and Methods. Cultures were induced with IPTG and arabinose or with only IPTG. Cell lysates were prepared from different culture volumes to obtain 5×10^6 cells at each time point. Quantities of Exg and PspA were obtained by optical scanning as described in Materials and Methods. **A**. Western blot analysis of the lysate samples with anti-Exg and PspA antibodies. **B**. Relative quantities of PspA with (▲) or without (□) arabinose induction. **C**, **D**. Relative quantities of pre-Exg (white columns) and Mat-Exg (gray columns) of lysate samples from the culture induced with (**C**) IPTG and arabinose or (**D**) only IPTG.

smaller ratio of 35/65, 20 hr after induction of the culture with only IPTG (Fig. 5D), an increase resulting likely from the leakage of expression of the non-induced recombinant *pspA* gene. A substantial increase in PspA was detected in the arabinose-induced culture 4 hr after induction, and the PspA level remained constant until the study ended at the 24th hr (Fig. 5B). However, PspA in the non-induced culture kept on increasing until the end point (24th hr) (Fig. 5B). The results showed that overexpression of PspA not only enhanced the expression of Exg, but also elevated its Pre/Mat RQ value, which was previously determined to be a small value of 20/80, designated the critical value (CV) of Exg [5], an index reflecting the maximum level of secretory Exg tolerable by its host cells without triggering dramatic cell death [4, 5].

Reduction of Membrane Depolarization and Damage with PspA Overexpression

To examine whether overexpression of PspA could reduce the membrane depolarization and damage induced by the hyperexpression of secretory Exg, cell samples were taken at different time intervals from JM101 (pM1VegGcexL-pspA) cultures induced with IPTG and arabinose or with only IPTG. The membrane depolarization and damage of the cells were detected by staining with fluorescence dyes, $DiBAC4_{(3)}$ and PI, respectively, and measured with flow cytometry. The fluorescence intensities of both dyes increased with time upon induced expression of Exg, indicating that the cytoplasmic membrane of the cells encountered both depolarization and damage. Dramatic increase in fluorescence intensities was shown to occur 6 hr after induction with IPTG alone (Fig. 6A and 6C), but not in the one induced with both IPTG and arabinose (Fig. 6B and 6D). Although an increase in both fluorescence intensities was then detected in the latter culture 8 hr after induction, the increments of both intensities were obviously less dramatic than those of the former culture (Fig. 6E and 6F). The results supported the conclusion that the overexpressed PspA protected the cytoplasmic membrane of *E. coli* against depolarization and damage resulting from hyperexpression of secretory Exg.

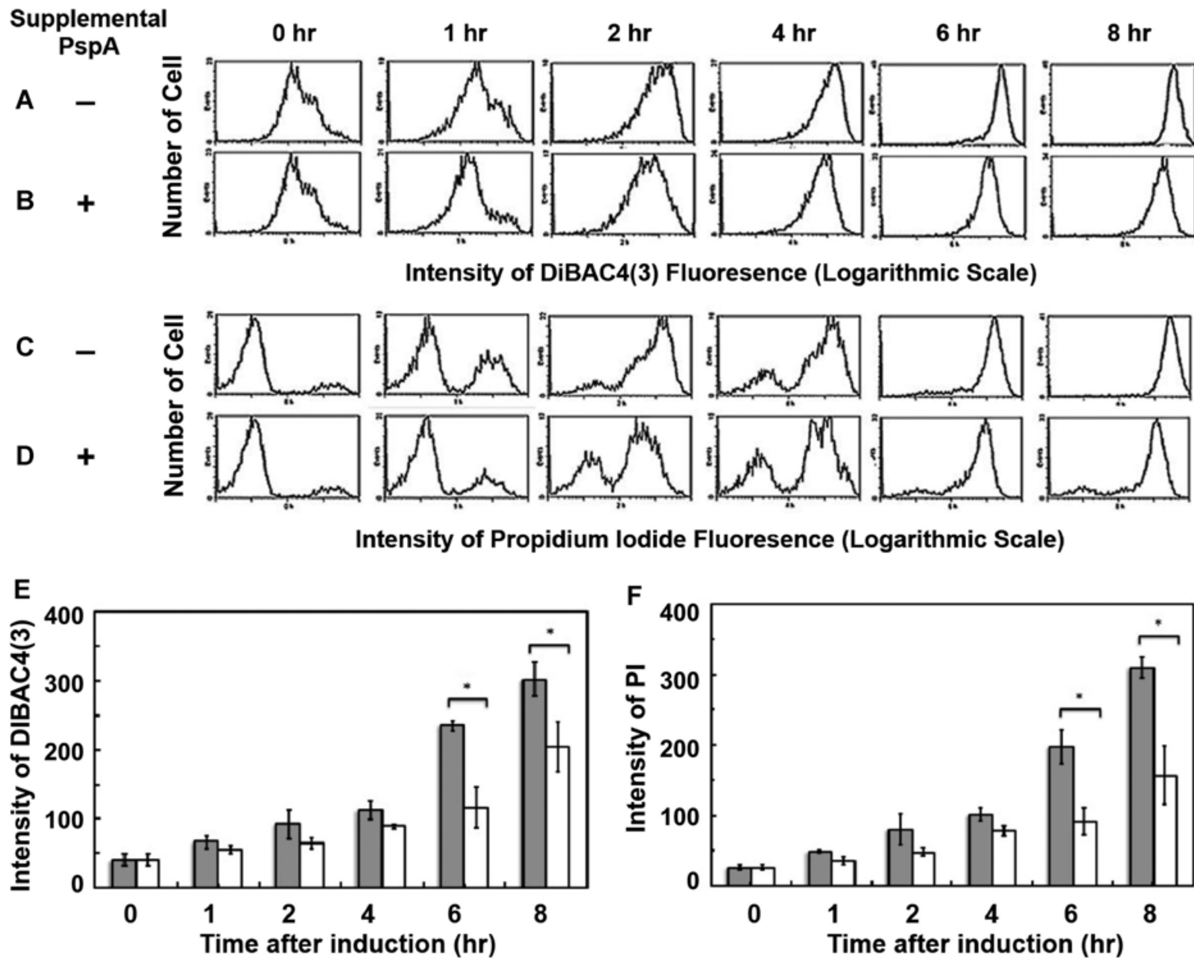

Fig. 6. Effects of PspA overexpression on membrane depolarization and damage in JM101 (pM1VegGcexL-pspA) producing secretory Exg.

JM101 (pM1VegGcexL-pspA) cultures were grown in 2× YT as described in Materials and Methods. Cultures were induced with IPTG and arabinose or with only IPTG. Samples taken from different time points after induction were diluted to 10^6 cells/ml, stained with 10 µg/ml $DiBAC_{4(3)}$ or 5 µg/ml PI, and assayed by flow cytometry as described in Materials and Methods. Each sample assay contained 20,000 events. **A**, **B**. Fluorescence intensities attained through staining with $DiBAC_{4(3)}$. **C**, **D**. Fluorescence intensities attained through staining with PI. **E**, **F**. Quantification of fluorescence intensities resulting from $DiBAC_{4(3)}$ and PI staining, respectively. The mean values ± SEM were derived from the results obtained in three different experiments. White columns show the culture induced with IPTG and arabinose; gray columns show the culture induced with only IPTG.

DISCUSSION

Extracellular production of heterologous proteins has many advantages over intracellular synthesis, including simplified downstream processing, enhanced biological activity, higher product stability and solubility, and N-terminal authenticity of the expressed protein. In addition, recombinant protein purification is simpler, thereby reducing the production costs. Despite some successful examples, excretory production of recombinant protein in *E. coli* remains problematic. The most frequently encountered problems are (1) incomplete secretion of recombinant protein, (2) insufficient capacity for secretion of overexpressed recombinant protein, (3) the death of host cells, (4) proteolytic degradation of the product, and (5) low amounts of recombinant protein released to the culture medium. We have employed the excretory approach to express a variety of heterologous proteins in *E. coli* [4, 5, 11, 20–22]. Regardless of the origin, composition, and size, a heterologous protein produced by secretion is capable of triggering cell death when it is expressed at high levels [4, 11, 21]. We have shown that hyperexpression of the *cex* gene resulted in "saturated translocation" of Pre-Exg in the Sec pathway, thereby interfering with the export of homogenous proteins and leading to cell death [4]. We then proposed the use of a ratio of Pre/Mat RQ, 20/80, designated the critical value, as a guide to attain the maximum expression of Pre-Exg [5]. A ratio of 18/82 was then obtained in strain JM101 (pM1VegGcexL), which could produce 50% higher excreted Exg than the previous best strain, JM101 (placUV5par8cex), offering a low Pre/Mat

RQ value of 10/90 [5]. However, when the ratio of Pre/Mat RQ was above the CV of 20/80, rapid cell death was triggered [4, 5].

PspA has been shown to be involved in the stress responses towards secretory expression of recombinant proteins, especially when the translocation machinery is blocked [2, 18]. We thus investigated the effects of hyperexpression of secretory Exg on the expression of endogenous PspA and the possible application of PspA in recombinant protein production. Hyperexpression of secretory Exg in strain JM101 (ptacIQpar8cex) resulted not only in dramatic cell death [4], but also suppression of endogenous PspA expression (Fig. 2). Overexpression of PspA in strain JM101 (ptacIQpar8cex-pspA) resulted in only slight improvement in the overall excreted Exg activity, when compared with its counterpart expressing just endogenous PspA (Fig. 3). However, when PspA was overexpressed in strain JM101 (pM1VegGcexL-pspA), the excreted Exg was enhanced significantly to 1.65 U/ml, which was 80% higher than that produced by its counterpart without PspA overexpression. Despite this remarkable difference between the two cultures, their viable cell counts were not largely different (Fig. 4A and 4B), suggesting that the overexpressed PspA functioned well to minimize the rapid cell death triggered by increased secretion of Exg observed in our previous studies [4, 11]. The cell-associated Exg activity in strain JM101 (pM1VegGcexL-pspA) was also shown to achieve better yields, of which the highest level was over twice as much as that produced without PspA overexpression (Fig. 4). The excreted Exg was further increased to 4.5 U/ml when rich medium MTB was used to attain a high cell density. The results illustrate the effectiveness of the combined application of PspA overexpression and high cell-density fermentation in excretory production of heterologous proteins.

In the JM101 (pM1VegGcexL-pspA) strain induced with both IPTG and arabinose, the overexpressed PspA resulted, notably, also in an improved CV of Exg, attaining the largest ratio of 45/55 (Fig. 5C), which is a lot higher than the previously identified value of 20/80 [5]. Despite the largely increased CV, thus reflecting a much elevated level of total Exg expression (Fig. 4D), the cell viability remained high (ca. 10^8 cells/ml) throughout the time-course study (Fig. 4A). The results suggested that the tolerable level of Pre-Exg in the cells increased with PspA overexpression. On the other hand, reduction of membrane depolarization and damage was observed 6 h after post-induction with PspA overexpression (Fig. 6E and 6F). It was postulated that PspA overexpression resulted in an elevated membrane potential, thereby preventing membrane damage and enhancing the tolerance of the cells towards Pre-Exg. These beneficial effects might not only enable the induced JM101 (pM1VegGcexL-pspA) culture to express higher levels of total Exg, but also facilitate the culture to secrete, and hence, excrete large quantities of Exg (Fig. 4C).

Collectively, our findings reported in this paper support the feasibility of metabolic engineering of PspA to derive a viable approach to enhance the excretory production of recombinant proteins. PspA overexpression was shown to be able to substantially improve the tolerance of *E. coli* towards Pre-Exg, from the previously reported small CV ratio of 20/80 [5] to the highest attainable value of 45/55 reported in this study (Fig. 5C). Therefore, the combination of metabolic engineering and construct optimization provides a practical approach for the development of the best possible recombinant *E. coli* strains for secretory/excretory production of heterologous proteins.

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