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An Efficient Secretion of Type I Secretion Pathway-Dependent Lipase, TliA, in *Escherichia coli*: Effect of Relative Expression Levels and Timing of Passenger Protein and ABC Transporter

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Abstract An ABC transporter apparatus of the Gram-negative bacterial type I secretion pathway can be used as a secretory protein expression system in *Escherichia coli*. Four types of coexpression systems for the *Pseudomonas fluorescens* lipase gene, *tliA*, and its cognate ABC transporter gene cluster, *tliDEF*, were constructed. When the relative expression levels were changed by adding different concentrations of IPTG, the secretion (16.9 U/ml of culture) of TliA in *E. coli* [pTliDEFA-223+pACYC184] was significantly higher than *E. coli* [pKK223-3+pTliDEFA-184] secreting the lowest level of TliA (5.2 U/ml of culture). Maximal accumulation of the lipase secreted occurred in the mid-exponential phase, implying that the efficient protein secretion *via* an ABC transporter was restricted only to actively growing cells. Finally, the secretion level of TliA in *E. coli* [pTliDEFA-223+pACYC184] was increased to 26.4 U/ml by inducing gene expression at the culture initiation time. These results indicate that a significant increase in the ABC transporter-dependent protein secretion can be achieved by simply controlling the relative expression levels between the ABC transporter and its passenger protein, even in the recombinant *E. coli* cells.

Key words: ABC transporter, *Escherichia coli*, lipase, type I secretion pathway

An ATP-binding cassette transporter (ABC transporter) of the Gram-negative bacterial type I secretion pathway has been used as a membranous apparatus for the secretory expression of recombinant proteins [2, 7, 20, 22]. In fact, the transplantation of a membranous apparatus of a protein secretion pathway into the nonpathogenic *E. coli* host has been one of the strategies to secrete recombinant proteins into the extracellular space [4, 24]. The ABC transporter apparatus of the type I pathway consists of only three protein components: ATP-binding protein (ABC protein), membrane fusion protein (MFP), and outer membrane protein (OMP) [3]. Therefore, the transplantation and/or reconstitution of whole gene clusters of ABC transporters into *E. coli* are relatively simple. Moreover, the distinct C-terminal signal sequence for type I secretion does not interfere with the endogenous Sec-dependent secretion pathways that are concerned with the export of most of the periplasmic and the outer membrane proteins in *E. coli* [3, 5, 18, 20]. The ABC transporter, particularly the HlyB/HlyD/TolC transporter, has been successfully applied for secretion of many recombinant proteins in *E. coli*, including single-chain Fv antibody [6], vaccine antigen [23], and human interleukin [9].

In addition to the relatively well-studied substrate specificity of the ABC transporter for the secretion of passenger protein [3, 5, 20], there may be other factors affecting the efficient secretion of passenger protein in the recombinant *E. coli* host. For example, the total amount of functional ABC transporter located in the cell membranes and the relative expression rates of passenger protein and ABC transporter may influence the heterologous secretion of passenger proteins in *E. coli*. Here, we describe the construction of coexpression systems for efficiently secreting the *P. fluorescens* lipase (TliA) via the type I secretion pathway in *E. coli*. We examined the effects of relative expression levels of passenger protein, TliA, and its cognate ABC transporter, TliDEF, on the secretion of TliA. The effect of expression timing of TliA and TliDEF was also examined. The secretion level of passenger protein, TliA, was increased about 5.1-fold by optimizing the coexpression system of the passenger protein and ABC transporter.

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Table 1. Oligonucleotide primers used for PCR amplification.[a]

Primer sequences (5'→3')	Genes to be amplified
GCCGAATTCATGTCGTTTGGTTCAAGGAAG (EcoRI) AGCAAGCTTATGAACCGCCGATAATCCCGTC (HindIII)	*tliDEFA* subcloned into pKK223-3
GCCGAATTCATGTCGTTTGGTTCAAGGAAG (EcoRI) AGCAAGCTTAATTGGCCGGAGATTGTGTGG (HindIII)	*tliDEF* subcloned into pKK223-3
GCCGAATTCATGGGTGTATTTGACTACAAG (EcoRI) AGCAAGCTTATGAACCGCCGATAATCCCGTC (HindIII)	*tliA* subcloned into pKK223-3
ATTGATATCCTCCTGATCGGGGGTGCGGGC (EcoRV) ATTGATATCATGAACCGCCGATAATCCCGTC (XhoI)	*tliDEFA* subcloned into pACYC184
ATTGATATCCTCCTGATCGGGGGTGCGGGC (EcoRV) ACGCTCGAGAATTGGCCGGAGATTGTGTGG (XhoI)	*tliDEF* subcloned into pACYC184
ATTGATATCACAAGAGAGACAAGACAATGG (EcoRV) ATTGATATCATGAACCGCCGATAATCCCGTC (XhoI)	*tliA* subcloned into pACYC184

[a]Restriction sites are underlined and the names of the restriction enzymes are in parentheses.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and DNA Manipulation

E. coli XL10-Gold (Stratagene, La Jolla, CA, U.S.A.) was used as a host strain for DNA manipulation and gene expression. Plasmids pKK223-3 (Amersham Pharmacia, Piscataway, NJ, U.S.A.) and pACYC184 (New England Biolabs, Beverly, MA, U.S.A.) were used as vectors. All DNA manipulations, including restriction endonuclease digestion, ligation, transformation, and agarose gel electrophoresis, were carried out by standard procedures [21]. All restriction enzymes, DNA-modifying enzymes, and related reagents used for DNA manipulation were purchased from Takara Shuzo (Shiga, Japan), Solgent (Daejeon, Korea), or Sigma (St. Louis, MO, U.S.A.).

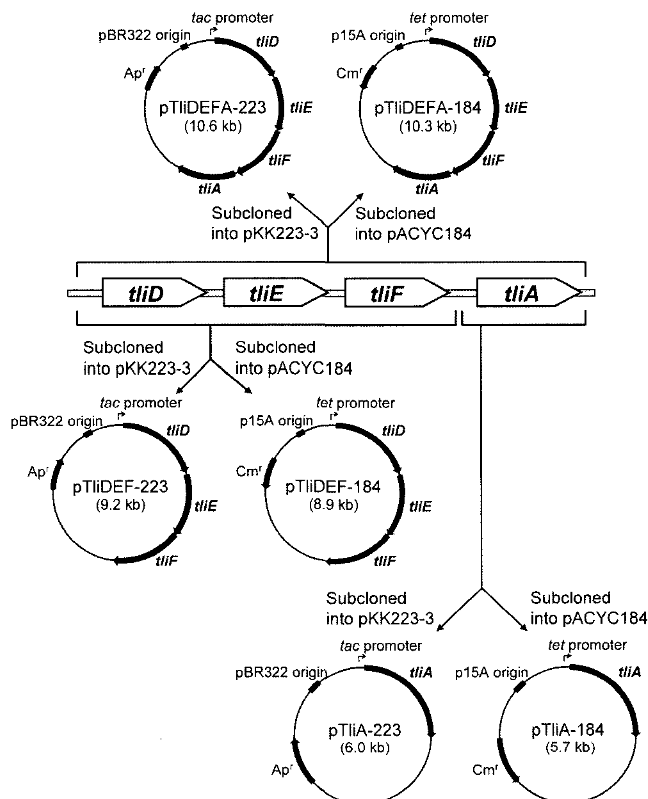

Fig. 1. Schematic diagram to construct the six recombinant plasmids used in this study.

Abbreviations: Ap^r, ampicillin resistance gene; Cm^r, chloramphenicol resistance gene.

Construction of Plasmids and Coexpression Systems

To subclone *tliDEFA*, *tliDEF*, and *tliA* into pKK223-3 and pACYC184 from the plasmid pTOTAL [1], PCR amplification was performed using the primer sets shown in Table 1. The PCR was carried out with an automatic thermal cycler (Bio-Rad, Hercules, CA, U.S.A.) for 30 cycles consisting of 94°C for 1 min, 58°C for 1 min, and 72°C for 6 min. The PCR products were digested with EcoRI and HindIII to construct pTliDEFA-223, pTliDEF-223, and pTliA-223, and with EcoRV and XhoI to construct pTliDEFA-184, pTliDEF-184, and pTliA-184 (Fig. 1). Each restriction enzyme-digested DNA fragment was gel-purified using a QIAGEN kit (QIAGEN, Hilden, Germany) and then ligated with the same enzyme-digested pKK223-3 and pACYC184, respectively. *E. coli* cells were co-transformed with each

Table 2. Coexpression systems of *tliA* and *tliDEF* in *E. coli*.[a]

Coexpressed plasmids	*tac* promoter-controlled gene(s)	*tet* promoter-controlled gene(s)
pTliDEFA-223+pACYC184	*tliDEFA*	-
pTliA-223+pTliDEF-184	*tliA*	*tliDEF*
pTliDEF-223+pTliA-184	*tliDEF*	*tliA*
pKK223-3+pTliDEFA-184	-	*tliDEFA*

[a]Coexpression systems of *tliA and tliDEF* were constructed by co-transforming *E. coli* XL10-Gold with each combination of plasmids.

combination of six resultant plasmids and control plasmids (Table 2).

Coexpression of *tliA* and *tliDEF* in *E. coli*

Luria-Bertani (LB) medium, supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml), was used for cultivation of the recombinant *E. coli*. All the *E. coli* cultures were carried out in 250-ml flasks containing 175 ml of LB medium in a shaking incubator at 25°C and 180 rpm. The culture temperature of 25°C was selected because psychrotrophic *P. fluorescens* has generally growth temperature-dependent characteristics and the TliDEF transporter used in this study functions optimally at temperatures below 30°C, particularly at 25°C [1, 13]. First, *E. coli* cells harboring the four types of coexpression systems were induced to express different levels of TliA and TliDEF by adding 0.01, 0.1, and 1 mM concentrations of IPTG at the cell density (OD_{600}) of 0.8. After the cultures were further grown for 12 h, the lipase secreted to the extracellular medium was measured. Second, the four types of *E. coli* cells were induced with 0.01 mM IPTG at 0.8 OD_{600} and the culture samples were collected at different time points. The time-courses of cell growth and TliA secretion were determined. Third, to examine an optimal expression timing for the efficient TliA secretion, *E. coli* [pTliDEFA-223+pACYC184] cells were induced with 0.01 mM IPTG at different OD_{600} of 0.03, 0.7, 1.0, 1.5, 2.0, and 3.0, respectively. In all the experiments, the culture supernatants were separated from the cell pellets by centrifugation at 13,000 ×*g* for 10 min and used for assaying the extracellular lipase activity.

Assay of Lipase Activity

Lipase activity was measured by the spectrophotometric method using *p*-nitrophenyl palmitate (*p*NPP) as a substrate. Ten mM *p*NPP dissolved in acetonitrile was mixed with ethanol and 50 mM Tris-HCl (pH 8.5) to a final ratio of acetonitrile:ethanol:Tris-HCl buffer to 1:4:95 (v/v/v). The reaction was started by adding 50 μl of culture supernatant to 200 μl of reaction mixture at 45°C, and absorbance at 405 nm was monitored with a microplate reader (Bio-Rad, Hercules, CA, U.S.A.) for 20 min. One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min.

Results

Construction of Coexpression Systems of *tliA* and *tliDEF* in *E. coli*

We developed four different types of coexpression systems of *tliA* and *tliDEF* to examine the effect of relative

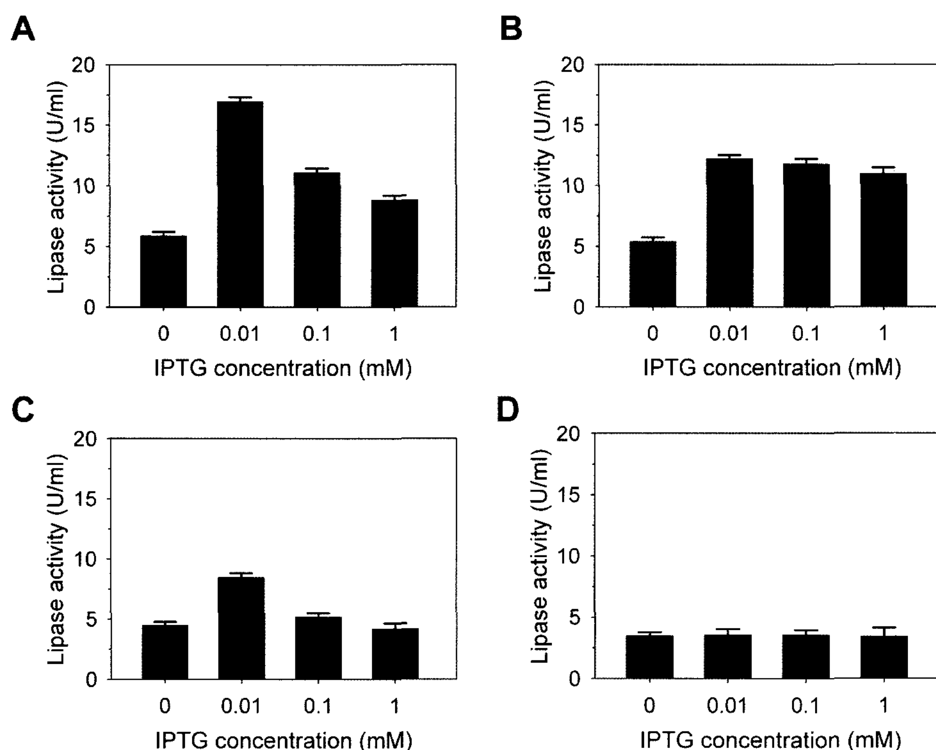

Fig. 2. Effect of relative expression levels of *tliA* and *tliDEF* on TliA secretion in (**A**) *E. coli* [pTliDEFA-223+pACYC184], (**B**) *E. coli* [pTliA-223+pTliDEF-184], (**C**) *E. coli* [pTliDEF-223+pTliA-184], and (**D**) *E. coli* [pKK223-3+pTliDEFA-184].
The cultures at OD_{600} of 0.8 were induced with different concentrations of IPTG.

expression levels of the ABC transporter and the passenger protein. First, six plasmids harboring *tliA* and/or *tliDEF* were constructed using two replicon-compatible vectors, pKK223-3 (the replicon pBR322) and pACYC184 (the replicon p15A) (Fig. 1): pKK223-3 derived from pBR322 is a medium copy number plasmid under the control of an IPTG-inducible *tac* promoter, and pACYC184 is a low copy number plasmid under the control of the weak constitutive *tet* promoter [17]. The four coexpression systems of *tliA* and *tliDEF* were then prepared by co-transforming *E. coli* cells with each combination of plasmids as shown in Table 2. Among the four expression systems, the pKK223-3-based genes were additionally varied by changing the concentration of IPTG added, whereas the pACYC184-based genes were constitutively expressed irrespective of the concentration of IPTG.

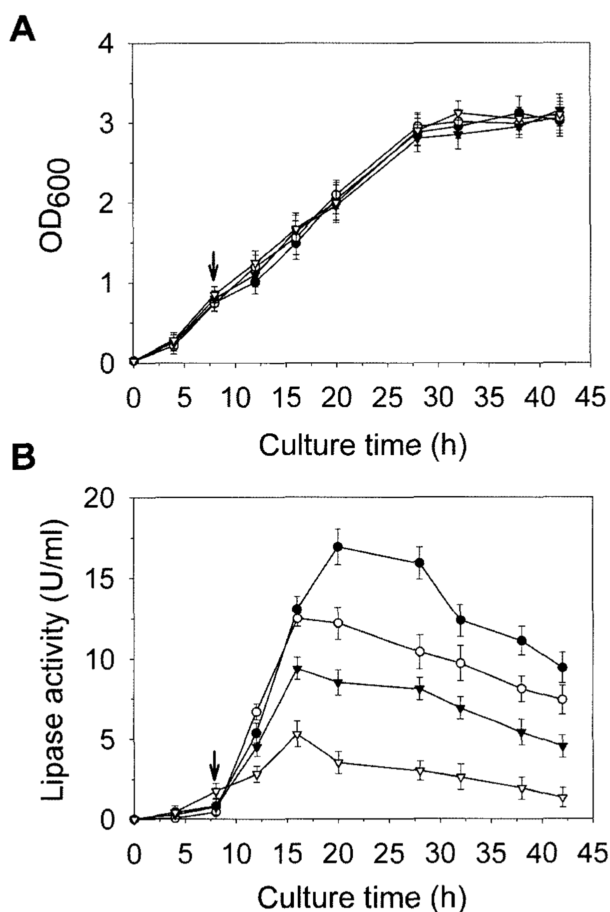

Fig. 3. Time-course profiles of (**A**) cell growth and (**B**) TliA secretion in the *E. coli* cells harboring the *tliA*/*tliDEF* coexpression systems.

The cultures were induced with 0.01 mM IPTG at OD_{600} of 0.8. Symbols: (●), *E. coli* [pTliDEFA-223+pACYC184]; (○), *E. coli* [pTliA-223+pTliDEF-184]; (▼), *E. coli* [pTliDEF-223+pTliA-184]; (▽), *E. coli* [pKK223-3+pTliDEFA-184]. The time point of IPTG induction is indicated by an arrow. The error bars represent the standard deviation calculated from three independent experiments.

Effect of Relative Expression Levels on TliA Secretion

The *E. coli* cells harboring each coexpression system were induced at OD_{600} of 0.8 with different concentrations of IPTG (0, 0.01, 0.1, and 1 mM). When the *tliA* and/or *tliDEF* genes were regulated by a *tac* promoter, the highest extracellular lipase activity was achieved in the culture supernatant by adding IPTG to a final concentration of 0.01 mM (A, B, and C in Fig. 2). *E. coli* [pKK223-3+pTliDEFA-184] showed almost the same extracellular lipase activity, regardless of IPTG concentration (Fig. 2D). Thus, a 0.01 mM IPTG concentration was chosen for induction in subsequent experiments.

We determined the temporal profiles of cell growth (Fig. 3A) and TliA secretion (Fig. 3B). Among the four types of *E. coli* cells, *E. coli* [pTliDEFA-223+pACYC184] showed the highest extracellular lipase activity: The extracellular lipase activity (16.9 U/ml) was approximately 3.3-fold higher than that (5.2 U/ml) of *E. coli* [pKK223-3+pTliDEFA-184] showing the lowest lipase activity. Interestingly, the lipase secreted from all the recombinants began to decrease significantly after the cell density reached OD_{600} of about 2, which corresponds to the mid-exponential growth phase.

Effect of Expression Timing of *tliA* and *tliDEF* on TliA Secretion Level

To examine the effect of expression timing of *tliA* and *tliDEF* on TliA secretion, *E. coli* [pTliDEFA-223+pACYC184] cells that exhibited the highest level of lipase secretion were induced with 0.01 mM IPTG at OD_{600} of 0.03, 0.7, 1.0, 1.5, 2.0, and 3.0, respectively. All the *E. coli* cultures showed similar growth profiles, irrespective of IPTG induction timing, implying that there was no harmful effect of different timing of IPTG induction on bacterial growth (Fig. 4A). However, the TliA secretion profiles were significantly affected by IPTG induction timing (Fig. 4B). As the cultures were induced earlier with 0.01 mM IPTG, the extracellular lipase activity was higher. Finally, the highest extracellular lipase activity (26.4 U/ml) was obtained when IPTG was added initially. The extracellular lipase activity at this point was 3.8-fold higher than that (6.9 U/ml) of the IPTG uninduced culture and 5.1-fold higher than that (5.2 U/ml) of *E. coli* [pKK223-3+pTliDEFA-184] exhibiting the lowest extracellular lipase activity among the four types of coexpression systems.

As shown in Fig. 3, the extracellular lipase activity also started to decrease after OD_{600} of approximately 1.5 to 2, which corresponds to the mid-exponential growth phase, regardless of IPTG induction timing (Fig. 4). Moreover, IPTG induction at the OD_{600} of 2 and 3, corresponding to mid- or late-exponential growth phase, could not reverse the decrease of the lipase secreted. These results suggest that the secretion of passenger protein (*i.e.*, TliA lipase) via

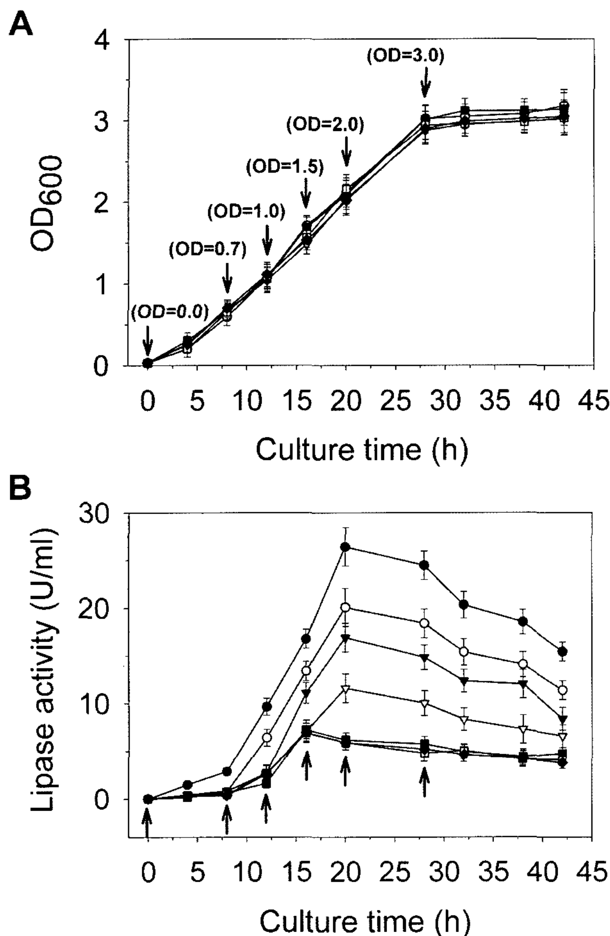

Fig. 4. Effect of expression timing of *tliA* and *tliDEF* on (**A**) cell growth and (**B**) TliA secretion in *E. coli* [pTliDEFA-223+pACYC184].

The *E. coli* cells were grown at 25°C and induced with 0.01 mM IPTG at different OD_{600} of 0 (●), 0.7 (○), 1.0 (▼), 1.5 (▽), 2.0 (■), and 3.0 (□). The uninduced culture (◆) was used as a negative control. The arrows indicate the time points of IPTG induction. The error bars represent the standard deviation calculated from three independent experiments.

the type I secretion pathway (*i.e.*, TliDEF ABC transporter) is closely associated with the cell growth phase.

Discussion

Among many strategies for extracellular expression of recombinant proteins, an ABC transporter apparatus of the Gram-negative bacterial type I secretion pathway has received considerable attention. Although there have been many successful examples in secreting various proteins and peptides with the type I secretion apparatus [2, 7, 20], correlation of the expression level between passenger protein and ABC transporter has not yet been examined in detail. In fact, there has been comparatively little progress in optimizing the type I secretion apparatus-based expression systems for improved extracellular production of recombinant proteins. In the present study, we investigated whether the relative expression level and the expression timing have an influence on the secretion of passenger protein by the ABC transporter. An extracellular lipase (TliA) and its ABC protein-type secretionary apparatus (TliDEF) of *P. fluorescens* were selected as a model system, and four types of coexpression systems were then constructed and examined. The results showed that the control of relative expression levels and expression timing between the ABC transporter apparatus and its target passenger protein could be a useful practical method to simply increase the ABC transporter-mediated secretion of recombinant passenger protein in *E. coli*.

As compared with *E. coli* [pKK223-3+pTliDEFA-184] that expresses equal levels of TliA and TliDEF, *E. coli* [pTliA-223+pTliDEF-184] appeared to have the same expression level of TliDEF transporter and the higher expression level of TliA. When compared, the relatively higher expression level of TliA induced a relatively higher amount of TliA secreted by an equal number of TliDEF transporter that may be anchored in the cell membranes (Figs. 2B, 2D, and 3). Practically similar results were obtained from both *E. coli* [pTliDEFA-223+pACYC-184] and *E. coli* [pTliDEF-223+pTliA-184], wherein they differed only in the expression levels of TliA (Figs. 2A, 2C, and 3). These results indicate that the relatively higher expression levels of the ABC transporter and/or passenger protein can increase the secretion level of passenger protein. However, the excessive overexpression of the ABC transporter and/or passenger protein caused a significant decrease in the secretion levels of passenger protein; most probably because of insoluble aggregates of passenger protein formed by itself and/or with membraneous ABC transporter [12, 14]. The secretion levels of TliA in *E. coli* cells harboring *tac* promoter-regulated coexpression systems were somewhat lower at higher than 0.01 mM IPTG concentrations (Fig. 2). In particular, the extent of decrease in TliA secretion levels was significantly more in *E. coli* [pTliDEFA-223+ pACYC-184] (Fig. 2A) than in *E. coli* [pTliA-223+ pTliDEF-184] (Fig. 2B). These data reflect that the excessive overexpression of the ABC transporter and/or passenger protein might block the translocation pathway and prevent the anchoring of the ABC transporter into the cell membranes. Therefore, the balanced increase in the expression levels of ABC transporter and passenger protein within some undefined limit is regarded to be important for increasing the secretion level of passenger protein.

The extracellular accumulation of TliA secreted by TliDEF was maximal at OD_{600} of about 2, corresponding to the mid-exponential growth phase (Fig. 3). Previous reports have shown that the secretory expression of proteins via the type I secretion system in native bacteria, particularly those of RTX toxins such as α-hemolysin of

E. coli and Apx toxins of *Actinobacillus* sp., was dependent on the bacterial growth phase [11, 19]. Extracellular accumulation of α-hemolysin and hybrid proteins fused to the C-terminal secretion signal was also found in "the growing cells" during the early- and mid-exponential growth phases of recombinant *E. coli* and *Salmonella* sp. [8, 9, 15]. The mRNA expression and the hemolytic activity of Apx toxin-secreting *Actinobacillus* sp. were maximal at the late-exponential and early-stationary growth phases, and they were rapidly decreased after reaching maximum levels [11]. Although the mechanisms related to the growth phase-mediated regulation of type I secretion have not yet been fully elucidated, the lipase secretion by the TliDEF apparatus of the type I secretion pathway in this study appeared to be clearly associated with the growth phase of recombinant *E. coli* cultures. A clear understanding of growth-phase dependency will serve as a guidance in making a factual determination of when the type I secretion apparatus-based bacterial cultures should be stopped in order to maximize the extracellular production of passenger protein.

The higher levels of extracellular TliA were achieved when the cultures of *E. coli* [pTliDEFA-223+pACYC-184] were induced as early as possible (Fig. 4B). The host cell growth was not directly affected by the expression and the subsequent ABC transporter-mediated secretion of functionally active lipase (Fig. 3A) and also by the addition of IPTG at various time points (Fig. 4A). Because the secretion of TliA via TliDEF occurred in the growing cells and might be continuously maintained to the mid- or late-exponential growth phase, a much longer period for expression and secretion of TliA therefore seemed to result in accumulation of more secreted TliA.

Besides the control of relative expression levels and expression timing of the ABC transporter and passenger protein in this study, some advances in the type I secretion-based extracellular expression of recombinant proteins were made previously by controlling culture conditions such as dissolved oxygen and pH [10, 19] and by genetically modifying expression plasmids in copy number [10] and codon usage [16]. Although the data on an oxygen-dependent secretory production of passenger protein are still preliminary, the culture conditions related to oxygen were also critical for the increase of TliA secretion level in our expression systems: a higher supply of air to cultures (*e.g.*, a higher agitation speed and a smaller culture volume) significantly decreased the TliA secretion level (data not shown). We are now in a process to investigate the cultivation parameters to further optimize the type I secretion of passenger protein *via* the ABC transporter in recombinant *E. coli* and other Gram-negative bacterial hosts. This report shows the first clear example to increase the secretion efficiency by controlling the relative expression level between passenger protein and the ABC transporter, and hopefully will serve as an experimental guide for extracellular production of recombinant protein using the ABC transporter.

Acknowledgments

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