Enhancement of the Efficiency of Secretion of Heterologous Lipase in *Escherichia coli* by Directed Evolution of the ABC Transporter System

Gyeong Tae Eom, Jae Kwang Song, Jung Hoon Ahn, Yeon Soo Seo, and Joon Shick Rhee

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, and Applied and Engineering Chemistry Division, Korea Research Institute of Chemical Technology, Daejeon, Republic of Korea

Received 8 September 2004/Accepted 7 January 2005

The ABC transporter (TliDEF) from *Pseudomonas fluorescens* SIK W1, which mediated the secretion of a thermostable lipase (TliA) into the extracellular space in *Escherichia coli*, was engineered using directed evolution (error-prone PCR) to improve its secretion efficiency. TliD mutants with increased secretion efficiency were identified by coexpressing the mutated *tliD* library with the wild-type *tliA* lipase in *E. coli* and by screening the library with a tributyrin-emulsified indicator plate assay and a microtiter plate-based assay. Four selected mutants from one round of error-prone PCR mutagenesis, T6, T8, T24, and T35, showed 3.2-, 2.6-, 2.9-, and 3.0-fold increases in the level of secretion of TliA lipase, respectively, but had almost the same level of expression of TliD in the membrane as the strain with the wild-type TliDEF transporter. These results indicated that the improved secretion of TliA lipase was mediated by the transporter mutations. Each mutant had a single amino acid change in the predicted cytoplasmic regions in the membrane domain of TliD, implying that the corresponding region of TliD was important for the improved and successful secretion of the target protein. We therefore concluded that the efficiency of secretion of a heterologous protein in *E. coli* can be enhanced by in vitro engineering of the ABC transporter.

Gram-negative bacteria use several strategies to secrete proteins across the inner and outer membranes into the extracellular environment. So far, five classes of secretion pathways have been identified in these bacteria (33). The type I secretion pathway (ABC transporter) examined in this study is rather different from the widespread type II secretion pathway. Proteins secreted by the type I pathway lack an amino-terminal (N-terminal) signal sequence. They cross both membranes without a periplasmic intermediate. The target proteins have an uncleaved carboxy-terminal (C-terminal) signal sequence containing several repeats of the glycine-rich sequence GG. Each of these repeats is followed by a hydrophobic amino acid, such as tryptophan or isoleucine, which is essential for the insertion of the protein into the cytoplasmic membrane. After their insertion, the C-terminal signal sequences are cleaved by a signal peptidase (33).

The ABC protein is an inner membrane protein composed of an N-terminal membrane domain containing six to eight transmembrane segments (14, 39) and a C-terminal ATPase domain (17, 26). The ABC transporter consists of three components, the ATP binding cassette (ABC) protein, the membrane fusion protein (MFP), and the outer membrane protein (OMP) (5). The ABC protein is an inner membrane protein composed of an N-terminal membrane domain containing six to eight transmembrane segments (14, 39) and a C-terminal ATPase domain (27). The ABC protein belongs to the well-characterized ABC protein superfamily, which includes eukaryotic and prokaryotic proteins related to the import or export of a wide variety of substrates, such as ions, antibiotics, sugars, amino acids, oligosaccharides, peptides, and proteins (21). It recognizes the C-terminal signal sequence of the target protein and supplies energy from ATP hydrolysis for secretion of the target protein (11, 25). MFP is exposed mainly to the periplasm and has one transmembrane segment anchored in the inner membrane (35). MFP connects the ABC protein and OMP during formation of the transport complex (23, 40). OMP is an outer membrane porin protein that forms a tunnel across the periplasm and the outer membrane (28).

*Escherichia coli* has long been the most versatile host for the production of recombinant proteins (3, 32). Generally, during cultivation of *E. coli* there are three compartments for accumulation of recombinant proteins. Secretion of recombinant proteins into the extracellular space has some advantages over production in the other compartments, the cytoplasm, and the periplasm. First, it does not result in the formation of inclusion bodies and the proteolytic degradation of recombinant proteins (32). Second, it simplifies separation and purification of recombinant proteins (32). Third, it allows continuous production of recombinant proteins since cells do not have to be lysed for recovery of the proteins (6, 24, 32). Moreover, the ABC transporter has additional advantages over other secretion pathways. Compared with other secretion machineries, the ABC transporter machinery consists of only three protein components, and thus its complexity is relatively low and it can be engineered and modified by molecular evolutionary approaches (19). Because the target proteins secreted by the ABC transporter have a distinct C-terminal signal sequence, their secretion generally does not interfere with the endogenous Sec-dependent export pathway concerned with the export of essential periplasmic and outer membrane proteins (13). Therefore, the ABC transporter has been used to secrete a number of recombinant proteins (6, 7, 22), such as the single-chain Fv antibody (13), vaccine antigen (15, 16, 36), and human interleukin (18), etc.
To make the ABC transporter a more efficient system for production of recombinant proteins, it is essential to enhance its secretion efficiency. To our knowledge, there have been no reports on the enhancement of the efficiency of secretion of the target protein by engineering the ability of the ABC transporter to secrete. We previously cloned all genes encoding the ABC transporter and showed that this transporter could mediate the extracellular secretion of ThiA in recombinant E. coli. (1). Here, we describe the use of directed evolution (29, 38) (error-prone PCR) to create TliD variants exhibiting an increased level of secretion of the heterologous ThiA lipase in E. coli.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. E. coli XL10-Gold (ΔmcrCΔmcrF) with a thiA gene and thiD gene (Strategene, San Diego, Calif.) was used as a host strain for DNA manipulation and gene expression. Plasmids pACYC184 (New England Biolabs, Beverly, Mass.) and pKK223-3 (Amersham Pharmacia, Piscataway, N.J.) were used as vectors. Plasmids pACYC184 and pKK223-3 were used for construction of pABCSK-ACYC harboring ABC transporter-encoding genes (i.e., a gene cassette composed of thiD, thiE, and thiF from Pseudomonas fluorescens SIK W1) and pTliA-PKK harboring a lipase-encoding gene (tlf from P. fluorescens SIK W1), respectively. Luria-Bertani (LB) medium was used for growth of recombinant E. coli. When necessary, ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) were added to the growth media.

DNA manipulation and sequencing. Standard recombinant DNA manipulation techniques were used for isolation of plasmids, restriction endonuclease digestion, ligation, transformation into E. coli, agarose gel electrophoresis of DNA, and purification of DNA fragments. 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.). DNA sequences were determined by cycle sequencing with an ABI PRISM BigDye primer cycle sequencing kit with AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, Calif.).

Construction of plasmids. PCR amplification of the thiD gene was performed with pTOTAL, which contained all four thi genes (thiD, thiE, thiF, and thiA), as the template DNA (1) and with two primers flanked by EcoRI or HindIII restriction sites (5′-ACAGAATTCTAGGTTTTAGACTACAAG-3′ and 5′-CAGAGGCTTCTGACCCAGGTAACG-3′). The amplified PCR products were digested with EcoRI and HindIII and then ligated with the same enzyme-digested pKK223-3 plasmid, resulting in plasmid pTliA-PKK. In order to facilitate the introduction of random mutations into only the thiD gene, plasmid pABCSK-ACYC was digested by inserting the same enzyme-digested pKK223-3 plasmid at the 5′ and 3′ ends of the thiD gene, respectively. The fusion gene was expressed in E. coli and was purified with nickel-nitrilotriacetic acid agarose resin (Qiagen). The fusion protein was used in experiments with the thiD gene.

Preparation of cell fractions. The recombinant E. coli cells were cultured at 25°C in 100 ml LB medium. The cells were harvested at the stationary phase (optical density at 600 nm [OD600] 4) and centrifuged at 4°C for 30 min at 13,000 × g for 10 min. The culture supernatants were concentrated fivefold using a Centricon (Millipore, Bedford, Mass.) for immunoblot analysis. In order to prepare cell lysates for assays of the intracellular lipase activity of the recombinant E. coli cells, cells were disrupted by freezing at an OD600 equivalent of 0.15 by lyzing with addition of E. coli turbidolytic solution (Genofocus, Daejeon, Korea) followed by incubation at 25°C for 30 min. One OD600 equivalent was the amount of cells or culture supernatant corresponding to 1 ml of culture at an OD600 of 1. For preparation of crude membrane fractions, the cells were disrupted with a French press (American Instrument Company, Silver Spring, Md.) and crude membrane pellets were collected by centrifugation at 4°C for 100,000 × g for 30 min.

Immunoblot analysis. For analysis of the level of secretion of ThiA in the recombinant E. coli cells, culture supernatants of recombinant E. coli cells were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then were electrophotographically transferred onto a nitrocellulose membrane. ThiA was detected with anti-ThiA antiserum as described previously (1). The level of expression of the TliD transporter in the recombinant E. coli cells was estimated by measuring the ThiD protein level by immunoblot analysis using the anti-ThiD antiserum. The gluthathione S-transferase (Amersham Pharmacia) gene and the six-histidine tag-encoding sequence were fused at the 5′ and 3′ ends of the thiD gene, respectively. The fusion gene was expressed in E. coli and was purified with nickel-nitrilotriacetic acid agarose resin (Qiagen). The fusion protein was used to obtain a mouse with the fused ThiD protein in Freund’s complete adjuvant (20). Membrane fractions of the recombinant E. coli cells were subjected to SDS-PAGE and then were electrophotographically transferred onto a nitrocellulose membrane. TliD was detected with anti-ThiD antiserum. The densities of signals on immunoblots were quantified with a GS710 calibrated imaging densitometer (Bio-Rad).

Assay of lipase activity. Lipase activity was assayed quantitatively by a spectrophotometric method using pNPP as a substrate (9). The pNPP was dissolved in acetonitrile at a concentration of 10 mM. Ethanol and 50 mM Tris-HCl (pH 8.5) buffer were subsequently added to this solution, in which the final ratio of acetonitrile to ethanol was 2:1 (vol/vol). Fifty microliters of culture supernatant or cell lysate was added to 500 μl of the pNPP solution and incubated at 45°C for 20 min, and the lipase activity was assayed by measuring the absorbance at 405 nm (the extinction coefficient of p-nitrophenol [pNP] was 18.1 cm2/μmol) with a microplate reader (Bio-Rad). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per min.

Assay of protease activity. Protease activity was measured by a slight modification of the method of Braun and Schmitz (8). Two hundred microliters of culture supernatant was added to 600 μl of a substrate solution containing 2.4% (wt/vol) azocasein in 50 mM potassium phosphate buffer (pH 7.5), and the mixture was incubated at 37°C for 30 min. The enzyme reaction was stopped by adding 600 μl of 10% trichloroacetic acid, and the mixture was allowed to stand for 1 h at 25°C. After centrifugation at 13,000 × g for 10 min, 800 μl of supernatant was collected and added to 300 μl of 10 N NaOH. The absorbance at 420 nm was measured with a spectrophotometer (Shimadzu, Kyoto, Japan). One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance per min by 1 under the experimental conditions.
RESULTS

Isolation of mutant ABC transporters exhibiting increased secretion of lipase. As a strategy to obtain ABC transporter variants showing increased efficiency in the secretion of recombinant proteins, we applied the directed evolution techniques to the *E. coli* system composed of the TliDEF transporter and the TliA lipase from *P. fluorescens*. In fact, we previously found that the TliDEF transporter can successfully function extracellularly to produce the TliA lipase from *E. coli* and *Pseudomonas* hosts (1, 2). One of the three component proteins of the ABC transporter, the ABC protein (TliD), plays an important role in secretion of target protein by recognizing the signal sequence of the target protein and subsequently supplying energy from ATP hydrolysis (11, 25). In addition, the binding of the ABC protein to the signal sequence of target proteins induces the sequential assembly of MFP and OMP (31), and the assembly of these components subsequently initiates the secretion of the target protein into extracellular space (4, 37). As a result, the interactions between the ABC protein and the target protein are the most decisive steps in successful secretion of a target protein. In this study, therefore, random point mutations were introduced only into the *tliD* gene by error-prone PCR, and the library of *tliD* variants was coexpressed with wild-type *tliA* in *E. coli* (Fig. 1).

Plasmid pABCSDK-ACYC containing the *tliDEF* genes was used as a template DNA in the first mutagenic PCR to introduce random point mutations only into the *tliD*-encoding region. *E. coli* cells carrying the *tliA* gene on a compatible plasmid, pTliA-PKK, were transformed with the resultant library of *tliD* variants. For library preselection, the cells were grown on semiquantitative indicator plates (LB-TB plates), and the capacities of different *tliD* variants to secrete the target lipase were estimated. From approximately 10,000 colonies, we selected 200 colonies that formed relatively larger halos from all members of the *tliD* library. The colonies selected were transferred to 1 ml LB medium in each well of 96-well deep plates and cultured at 25°C. The extracellular lipase activity of each clone was measured and compared with that of the clone harboring the wild-type *tliD* gene. While 20% of the clones showed lipase activity comparable to that of clones harboring the wild-type *tliD* gene, 50% of the clones showed an increase in the extracellular lipase activity of about 30 to 100% and 10% of the clones showed an increase in the extracellular lipase activity of more than 100%. The remaining 20% of the clones showed a decrease in the extracellular lipase activity of up to 50% (data not shown). Four of the clones showing the highest extracellular lipase activity were isolated. Plasmids encoding each mutant protein were isolated and pooled (equal amounts of all plasmids). We used this plasmid mixture as a template DNA in the second round of mutagenesis in order to further improve the efficiency of secretion. After the second round of PCR mutagenesis and two-step screening of 10,000 clones, however, no clones that showed higher extracellular lipase activity were identified. Consequently, we selected four clones from the first round of mutagenesis for further characterization. The plasmids of these clones were designated pT7, pT8, pT24, and pT35 (Fig. 2).

Characterization of selected ABC transporter variants. *E. coli* pTliA-PKK clones harboring the wild-type and mutant *tliD* genes were cultured in 100 ml LB medium at 25°C. The extracellular and intracellular lipase activities of each clone were assayed with culture supernatant and cell lysate, respectively (Table 1). Compared with the wild-type TliDEF, the selected TliD variants, T6, T8, T24, and T35, showed 3.2-, 2.6-, 2.9-, and 3.0-fold increases in the level of secretion of TliA, respectively (Fig. 3A). Immunoblot analysis was also used to estimate the level of secretion of TliA in the culture supernatant of each clone. As shown in Fig. 3B, extracellular TliA secretion by the *E. coli* pTliA-PKK clones expressing the mutant *tliD* genes was significantly increased. Based on the results shown in Fig. 3, introduction of a random mutation into the gene encoding the ABC protein TliD, a constituent of the ABC transporter cluster, could increase the level of secretion of TliA, a target protein of the ABC transporter cluster, in a recombinant *E. coli* host. Therefore, we concluded that the ABC transporter...
machinery, in particular the ABC protein, could be successfully engineered by directed evolution for improved secretion of a target protein.

In order to determine whether the improved ability to secrete the target protein TliA was due to a difference in the level of expression of the TliD transporter in the membrane of the E. coli host, an immunoblot analysis of the TliD protein was performed with membrane fractions of E. coli harboring the wild-type and mutant tliD genes (Fig. 4). The levels of expression of TliD in the membrane fraction were almost the same for all clones tested. Introduction of a mutation into the tliD gene, therefore, did not affect the level of expression of the TliD transporter in E. coli. This result indicates that the increased level of secretion of TliA was not due to the increased expression of TliD variants in the E. coli host but was due to enhancement of the variants’ ability to secrete.

Each of the four TliD variants had a single point mutation leading to one amino acid change in TliD. All the mutations were located in the predicted cytoplasmic regions in the membrane domain of TliD (Fig. 5). This result also indicates that the corresponding region of TliD may be the important site for affecting secretion of TliA into extracellular space.

**DISCUSSION**

The goal of this work was to enhance the ability of the ABC transporter to secrete its target protein. In this study, we demonstrated that introduction of a mutation only into the tliD gene encoding one of the three component proteins of the TliDEF transporter could increase the ability of this transporter to secrete. To isolate TliDEF variants in order to increase the amount of lipase secreted, we performed a two-step activity-based screening procedure involving an LB-TB indicator plate and a 96-well microtiter plate. First, a semiquantitative analysis of the lipase secreted on LB-TB plates by a number of E. coli cells harboring the plasmids was performed by comparing the sizes of lipolytic clear halos. After E. coli cells containing each TliD variant isolated from the first round of mutagenesis and screening were incubated on the LB-TB plate at 25°C for 46 h, the produced larger halos than the wild-type TliDEF. Colony 1, E. coli (pTliA-PKK, pABCSK-ACYC) (wild-type tliDEF gene); colony 2, E. coli (pTliA-PKK, pACYC184) (negative control); colony 3, E. coli (pTliA-PKK, pT6); colony 4, E. coli (pTliA-PKK, pT8); colony 5, E. coli (pTliA-PKK, pT24); colony 6, E. coli (pTliA-PKK, pT35).

**TABLE 1. Secretion of lipase by recombinant E. coli (pTliA-PKK) cells harboring the wild-type and mutant tliD genes**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Lipase activity (U ml⁻¹ OD₆₀₀⁻¹)</th>
<th>Secretion efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell lyase</td>
<td>Culture supernatant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTliA-PKK + pACYC184</td>
<td>9.6 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>pTliA-PKK + pABCSK-ACYC</td>
<td>11.6 ± 0.1</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>pTliA-PKK + pT6</td>
<td>5.7 ± 0.2</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>pTliA-PKK + pT8</td>
<td>6.1 ± 0.2</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>pTliA-PKK + pT24</td>
<td>5.8 ± 0.1</td>
<td>8.3 ± 0.4</td>
</tr>
<tr>
<td>pTliA-PKK + pT35</td>
<td>5.9 ± 0.3</td>
<td>8.5 ± 0.3</td>
</tr>
</tbody>
</table>

*a E. coli cells harboring the plasmids were grown at 25°C for 30 h in LB medium. The cells were harvested at the stationary phase (OD₆₀₀: 4).

*All experiments were carried out in triplicate.

*Secretion efficiency = lipase activity in culture supernatant/(lipase activity in culture supernatant + lipase activity in cell lysate) × 100.

*ND, not detected.
ber of TliDEF variants was carried out by comparing the sizes of clear halos. To make the size of a clear halo proportional to the amount of lipase secreted, we ensured consistent thickness and flatness of LB-TB indicator plates and determined the optimum incubation times (40 to 48 h) at which most of the library members exhibited a suitable range of halo sizes. Under the conditions used in this first screening step, we could reduce the number of candidate clones from approximately 10,000 to 200 by a 2% ratio. When the extracellular lipase activity of each clone was assayed in the second screening step based on the 96-well plate format, approximately 60% of the 200 clones showed an increase in extracellular lipase activity compared to the clones expressing wild-type TliD. This result indicates that LB-TB plate-based visual screening was effective for reducing the number of clones to be tested with the 96-well plate-based lipase assay.

Three factors are considered the important determinants of efficient secretion by an ABC transporter: (i) the intracellular amount of target proteins, which is mainly influenced by the total expression level, (ii) the total amount of ABC transporter in the membrane of E. coli, and (iii) the ability of the ABC transporter to secrete. In order to improve the last factor, tliA and tliDEF were subcloned into different plasmids, pKK223-3, which is a medium-copy-number plasmid under the control of the inducible tac promoter, and pACYC184, which is a low-copy-number plasmid under the control of a constitutive tet promoter, respectively. In this manner, the expression of the target protein in each member of the E. coli library could be maintained at levels much higher than those observed with the ABC transporter, and therefore, an increased ability of the ABC transporter that could continuously secrete the excess amount of target protein was successfully identified.

The DNA sequence analysis revealed that only single amino acid substitutions occurred in all the mutants selected and that they were located in the predicted cytoplasmic regions in the membrane domain of TliD. Meanwhile, when an additional mutation was introduced into the clones from the first round during the second round of mutagenesis and screening, we did not obtain any clones that showed higher extracellular lipase activity. Thus, it is certain that just one amino acid change in TliD of the TliDEF transporter can improve the ability of the TliDEF transporter to secrete the TliA target protein. Our results are comparable with the results of complementation mutagenesis of transport-deficient E. coli hemolysin A (HlyA) mutants (41). The secretion of HlyA is naturally mediated through an E. coli ABC transporter comprised of HlyB (ABC protein), HlyD (MFP), and TolC (OMP) (6). HlyB variants,
which could secrete the C-terminal signal sequence-defective HlyA mutant, also had single point mutations that changed one amino acid of the predicted cytoplasmic regions in the membrane domain of HlyB. Therefore, we believe that all the TliD mutations resulted in more efficient interaction of TliD variants with the C-terminal signal sequence of TliA and thus facilitated the secretion of TliA. However, the detailed mechanism and why mutation of TliD increased the ability of the TliDEF transporter to secrete TliA are still unclear.

In order to determine the effect of our TliDEF mutants on the efficiency of secretion of another target protein, we attempted to use TliDEF mutants to secrete the protease PrtA, which was encoded upstream of the tliDEF genes in the natural microbial host, *P. fluorescens* SIK W1 (1). The primary structure of the C-terminal signal sequences of PrtA bears little similarity to that of TliA; nevertheless, PrtA is secreted through the wild-type TliDEF transporter with an efficiency comparable to that of TliA secretion. When the amount of PrtA secreted by each of the TliDEF mutants was examined, the results showed that none of the TliDEF mutants exhibited comparable to that of TliA secretion. When the amount of PrtA secreted by each of the TliDEF mutants was examined, the results showed that none of the TliDEF mutants exhibited increased secretion of PrtA (Fig. 6). The fact that the mutations that resulted in the increase in lipase secretion did not influence the secretion of PrtA indicates that the TliDEF transporter recognizes the signal sequences of TliA and PrtA in different ways.

The ABC transporter has received some biotechnological attention as a system for production of recombinant proteins in *E. coli*. First, it is important to increase the efficiency of secretion of the target protein (for example, by improving the ability of the ABC transporter to secrete). In addition, increasing the amount of the functional ABC transporter located in the cellular membrane can be another way to increase the efficiency of secretion of a target protein. Based on our results, we concluded that the efficiency of secretion of the target protein was successfully increased by enhancing the ability of the ABC transporter to secrete through directed evolution techniques and not by increasing the amount of the functional ABC transporter. Therefore, we are now attempting to find an appropriate coexpression system to maximize the secretion of a functional target protein by modulating the level of ABC transporter expression in the membrane of *E. coli*.

In addition to the extracellular production of useful proteins in *E. coli*, the protein secretion system using the ABC transporter should also be a very efficient *E. coli* expression vector system for high-throughput screening. Foreign protein variants which are fused with a C-terminal signal sequence suited for the ABC transporter machinery can be secreted into the extracellular medium, although not all of them fold correctly into their functional forms. If a protein that has been difficult to screen in other expression systems can be secreted by the ABC transporter, it can be used more easily for high-throughput screening for directed evolution work. Now we are also investigating the usefulness of the ABC transporter system for activity-based high-throughput screening of a number of foreign protein variants.

**ACKNOWLEDGMENTS**

This work was supported in part by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (grant MG05-0103-3-4), Republic of Korea.

**REFERENCES**


