Journal of Bioscience and Bioengineering
VOL. 122 No. 3, 283–286, 2016

ELSEVIER

www.elsevier.com/locate/jbiosc

Alleviation of temperature-sensitive secretion defect of *Pseudomonas fluorescens* ATP-binding cassette (ABC) transporter, TliDEF, by a change of single amino acid in the ABC protein, TliD

Gyeong Tae Eom,[1,2] Joon Young Oh,[1] Ji Hyun Park,[1] Hye Jin Lim,[1,3] So Jeong Lee,[1] Eun Young Kim,[1] Ji-Eun Choi,[1] Jonggeon Jegal,[1,2] Bong Keun Song,[1,2] Ju-Hyun Yu,[1] and Jae Kwang Song[1,4,*]

Research Center for Bio-based Chemistry, Korea Research Institute of Chemical Technology (KRICT), 141 Gajeong-ro, Yuseong-gu, Daejeon 305-600, Republic of Korea,[1] Center for Industrial Chemical Biotechnology, Korea Research Institute of Chemical Technology (KRICT), 45 Jongga-ro, Jung-gu, Ulsan 681-802, Republic of Korea,[2] Department of Fine Chemical Engineering and Applied Chemistry, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 305-764, Republic of Korea,[3] and Department of Green Chemistry and Environmental Biotechnology, Korea University of Science and Technology (UST), 217 Gajeong-ro, Yuseong-gu, Daejeon 305-350, Republic of Korea[4]

Received 21 October 2015; accepted 28 February 2016
Available online 29 March 2016

An ABC transporter, TliDEF, from *Pseudomonas fluorescens* SIK W1, mediates the secretion of its cognate lipase, TliA, in a temperature-dependent secretion manner; the TliDEF-mediated secretion of TliA was impossible at the temperatures over 33°C. To isolate a mutant TliDEF capable of secreting TliA at 35°C, the mutagenesis of ABC protein (TliD) was performed. The mutated *tliD* library where a random point mutation was introduced by error-prone PCR was coexpressed with the wild-type *tliE*, *tliF* and *tliA* in *Escherichia coli*. Among approximately 10,000 colonies of the *tliD* library, we selected one colony that formed transparent halo on LB-tributyrin plates at 35°C. At the growth temperature of 35°C, the selected mutant TliD showed 1.75 U/ml of the extracellular lipase activity, while the wild-type TliDEF did not show any detectable lipase activity in the culture supernatant of *E. coli*. Moreover, the mutant TliD also showed higher level of TliA secretion than the wild-type TliDEF at other culture temperatures, 20°C, 25°C and 30°C. The mutant TliD had a single amino acid change (Ser287Pro) in the predicted transmembrane region in the membrane domain of TliD, implying that the corresponding region of TliD was important for causing the temperature-dependent secretion of TliDEF. These results suggested that the property of ABC transporter could be changed by the change of amino acid in the ABC protein.

[**Key words:** *Pseudomonas fluorescens*; ATP-binding cassette (ABC) transporter; ABC protein; Temperature-dependent secretion; Random mutagenesis; Lipase]

The type I protein secretion system in Gram-negative bacteria is mediated by the action of ATP-binding cassette (ABC) transporter, which comprised three independent polypeptides, named ABC protein, membrane fusion protein (MFP) and outer membrane protein (OMP) (1,2). The OMP forms a channel across the periplasm and the outer membrane (3), and the MFP with single transmembrane segment in the inner membrane is exposed to the periplasm (4–6). The ABC protein recognizes the signal sequence of the secreted protein and hydrolyzes ATP to provide energy for the protein translocation (7,8). For example, HlyB, an ABC protein for the secretion of *Escherichia coli* hemolysin, was composed of an N-terminal membrane domain containing six to eight transmembrane segments (9,10) and a C-terminal ATPase domain (11). Proteins secreted by the ABC transporter have an uncleaved C-terminal signal sequence containing several repeats of the glycine-rich sequences GGXGXD (12,13), an amphipathic α-helix region (14,15), and an extreme C-terminal motif (16).

The *Pseudomonas fluorescens* ABC transporter, TliDEF, mediated the secretion of thermostable lipase (TliA) and metalloprotease (PrtA) both in the homologous host (17) and in the heterologous *E. coli* host (18). An increase in the amount of TliA of the extracellular medium was achieved by applying the directed evolution method to the TliD-encoding gene (19). TliD mutants with increased secretion efficiency were isolated by screening the TliDEF-TliA library containing thousands of TliD mutants in *E. coli*. Such a change in the properties of ABC transporter has been also induced by amino acid substitutions in *E. coli* hemolysin transporter (20–22).

Unlike other ABC transporters involved in the gram-negative bacterial type I secretion system such as those of *Pseudomonas aeruginosa* (12), *Serratia marcescens* (23) and *Erwinia chrysanthemi* (24), only the TliDEF transporter secreted the target proteins in a temperature-dependent secretion manner (17,25). In other words, *P. fluorescens* and *E. coli* expressing the TliDEF transporter-encoding genes could not secrete the lipase TliA above 33°C at all. We supposed that the unique structure-function relationship of TliD, the ABC protein of TliDEF, in the inner membrane was involved in the temperature dependence of TliDEF.

In this study, we thus employed the random mutagenesis to create a library of TliD variants and finally obtained a mutant TliDEF capable of secreting TliA at 35°C. Then, we investigated the

* Corresponding author at: Research Center for Bio-based Chemistry, Korea Research Institute of Chemical Technology (KRICT), 141 Gajeong-ro, Yuseong-gu, Daejeon 305-600, Republic of Korea. Tel.: +82 42 860 7643; fax: +82 42 860 7049.
E-mail address: ajee@krict.re.kr (J.K. Song).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.02.013

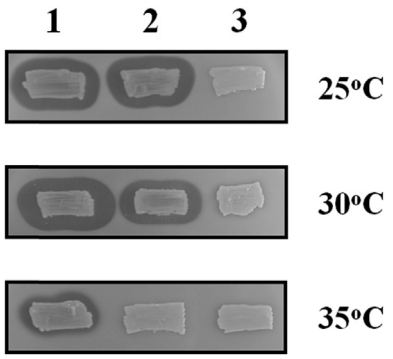

FIG. 1. Detection of extracellular lipase activity on LB-tributyrin agar plates. The clear zones around *E. coli* cells indicate that the TliA lipase was successfully secreted by the coexpressed TliDEF transporters. The *E. coli* cells were incubated on the LB-tributyrin plate at 25°C, 32°C, and 35°C, respectively. 1, *E. coli* [pTliA-PKK + pTMU1]; 2, *E. coli* [pTliA-PKK + pABCSK-ACYC]; 3, *E. coli* [pTliA-PKK + pACYC184].

mutation site of the isolated TliD mutant and compared the secretion capacity of TliA with the wild-type TliDEF according to the growth temperatures.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture medium *E. coli* XL10-Gold {*Δ*(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q} *ZΔM15* Tn10 (Tet^{r}) Tn5 (Kan^{r}) Amy]} (Stratagene) was used as a host strain for DNA manipulation and gene expression. Plasmids pACYC184 (New England Biolabs) was used as a control vector. Plasmid pTliA-PKK harbored the lipase gene (*tliA*) of *P. fluorescens* SIK W1 in the plasmid pKK223-3 backbone (19). Plasmid pABCSK-ACYC contained the ABC transporter gene cluster composed of *tliD*, *tliE* and *tliF* of *P. fluorescens* SIK W1 in the plasmid pACYC184 backbone (19). Two restriction enzyme sites, *Sac*I and *Kpn*I, were located at the 5′ and 3′ ends of *tliD* gene, respectively, to facilitate the introduction of random mutations into *tliD* gene of the plasmid pABCSK-ACYC.

Random mutagenesis and mutant library construction Random mutagenesis of *tliD* gene and construction of mutant TliDEF library were carried out as previously described (19). Two oligonucleotides, 5′-GGTCTAGATTTCAGTGCA ATTTATCTCTTCAA-3′ and 5′-ATTGGATCCCCGCCTGCTCAC-3′, were used as the forward and reverse primers for error-prone PCR to randomly mutate *tliD* gene (26). In order to obtain the desired level of mutation (0.5–2 nucleotide substitutions per 1 kb of gene), the conditions used for PCR random mutagenesis were optimized; a 100-μl reaction mixture contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 7 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 25 pmol of each oligonucleotide primer, 5 ng of template, and 5 U of Taq polymerase (Solgent, Korea). The PCR was performed with an automatic thermal cycler (Bio-Rad) for 30 cycles consisting of 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min. The mutagenic PCR products were digested with *Sac*I and *Kpn*I and were gel-purified by using a Qiagen kit (Qiagen). The purified PCR products were ligated with the same enzyme digested pABCSK-ACYC. *E. coli* cells harboring pTliA-PKK (*E. coli*/pTliAPKK) were transformed with the resulting ligated DNA and plated on LB agar plates containing 1% (v/v) tributyrin with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 50 μg/ml ampicillin, and 34 μg/ml chloramphenicol.

Screening procedure To screen the *tliD* mutants which could secrete TliA at 35°C, *E. coli* cells harboring the randomly mutated *tliD* gene and the native *tliE-tliF* genes were grown at 35°C on LB-tributyrin plates for 40 h. While *E. coli* cell coexpressing the wild-type *tliDEF* gene and the *tliA* gene could not form any clear halos on LB-tributyrin plates at 35°C, the selected *E. coli* cells coexpressing the mutated *tliD* gene and the native *tliE-tliF* gene did form clear halos on LB-tributyrin plate at 35°C. To confirm the secretion ability of the selected *tliD* mutant at 35°C, the recombinant *E. coli* cells were also grown on LB-tributyrin plate at 25°C, 30°C and 35°C.

Culture conditions After selecting the *tliD*-mutating *tliDEF* mutant which could secrete TliA at 35°C, the secretion level of TliA between the selected *tliD*-mutating *tliDEF* mutant and the wild-type *tliDEF* was compared at 20°C, 25°C, 30°C and 35°C. The recombinant *E. coli* cells were cultivated in 250-ml flasks containing 150 ml of LB medium at 20°C, 25°C, 30°C and 35°C at agitation speed of 250 rpm, and induced with 0.01 mM IPTG when cultivation was started. When necessary, ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) were added to the growth media. After cultivation, the culture supernatants were separated from the cell pellets by centrifugation at 13,000 ×*g* for 10 min. Lipase activity was measured quantitatively by pH titration of fatty acids liberated from olive oil as described previously (17). One unit of lipase activity was defined as the amount of lipase necessary to release 1 μmol of fatty acids per minute at 45°C and pH 8.5. Cell growth was monitored by measuring optical density of cultures at 600 nm (OD_{600}) with a spectrophotometer (Shimadzu, Japan). All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

When we tried to use the TliDEF to highly secrete the lipase TliA in *E. coli*, we found out that the secretion ability of the wild-type

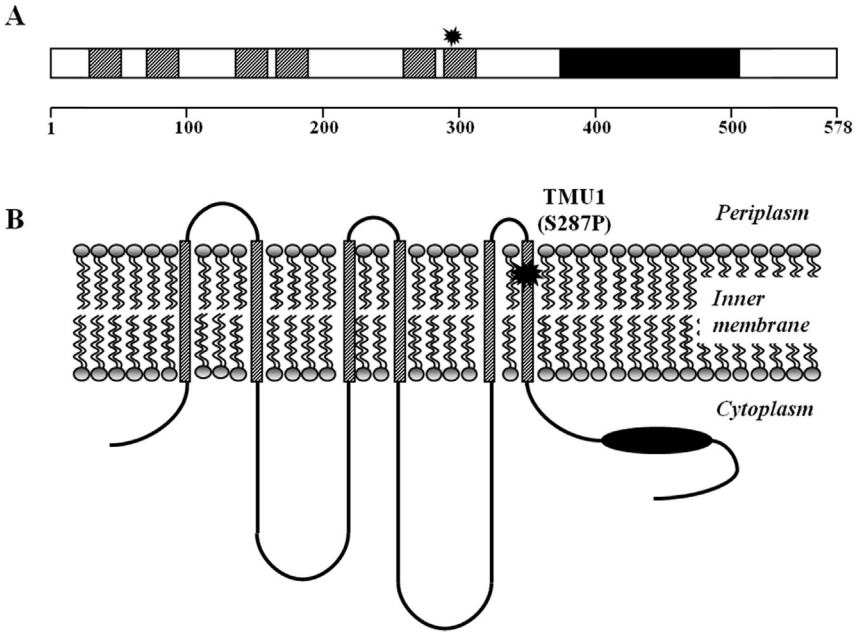

FIG. 2. Location of mutations on the TliD-encoding region. (A) Mutations in TliD marked on the primary structure of TliD. The bar represents the 578 amino acids of the TliD-encoding region, and the line at the bottom indicates the relative position of mutations. The shaded boxes represent the transmembrane segments, and the box in black indicates the ATP binding region. The asterisk represents the location of mutation. (B) Location of mutations on the secondary structure of TliD in the inner membrane of *E. coli*. The secondary structure of TliD is predicted by SOSUI program (http://sosui.proteome.bio.tuat.ac.jp). The black ellipse indicates the ATP binding region.