Chapter 19
Construction of *Eschericha coli-Staphylococcus* Shuttle Vector for EGFP Expression and Potential Secretion via Tat Pathway

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Abstract In this study, the potential for heterologous protein expression and secretion via twin-arginine translocation (Tat) pathway was investigated in *Escherichia coli* DH5α host using enhanced green fluorescent protein (EGFP) as model protein reporter. To construct the shuttle vector pBT2-ET-5X-EGFP, 17 kinds of PCR-amplified *5x-egfp* fragments were, respectively, cloned into plasmid pBT2-Peftu-Tat-EGFP and transformed into *E. coli* DH5α host. By SDS-PAGE, fluorescence microscope observation and flow cytometry analyzation, EGFP was expressed in an active form in the cells of *E. coli* DH5α, but failed to translocate to the culture medium.

Keywords *E. coli* DH5α · Enhanced green fluorescent protein (EGFP) · Twin-arginine translocation pathway · Plasmid pBT2

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19.1 Introduction

Compared to the general protein secretion (Sec) system [1], the twin-arginine translocation (Tat) system can deliver the properly folded proteins to the extracellular environment [2]. Thus, the Tat system can be used in gene engineering to secrete exogenous protein which cannot utilize the Sec system [3].

Green fluorescent protein (GFP) can be expressed in *Escherichia coli* as an ideal reporter [4], but whether it is correctly folded and transported across the plasma membrane depends on the choice of the Tat signal peptide sequence [2]. The wild-type GFP is sensitive to temperature, sometimes less expressed and weakly fluorescent in some cells [5], thus researchers have optimized GFP through site-directed mutagenesis. The enhanced green fluorescent protein (EGFP) is now a widely used GFP mutant with two independent substitutions (F64L, S65T), which has improved the fluorescence intensity as well as stability to be detectable for 16–24 h after excitation [6]. In this study, N-terminal modified EGFP was used as model reporter to study the Tat-dependent secretion pathway in *E. coli* DH5α host.

Shuttle vector has two different replication origins of two microbial plasmids, selection marker gene and a multicloning site, which can be replicated in two different organisms, and is usually used for cloning the amplified cloned gene. In this study, pBT2-ET-5X-EGFP, a shuttle vector of *E. coli* and *Staphylococcus* derived from a shuttle vector pBT2 [7], was constructed to study the expression and secretion of EGFP via Tat-pathway.

19.2 Materials and Methods

19.2.1 Bacterial Strain, Plasmids, and Growth Medium

The bacterial strain and plasmids used in this study are listed in Table 19.1, and all the recombinant DNA manipulations were carried out in *E. coli* DH5α host. *E. coli* DH5α was cultured at 37 °C in LB broth (0.5 % yeast extract, 1 % tryptone, and 1 % NaCl) supplemented with 100 μg/mL ampicillin for plasmid selection.

19.2.2 Preparation of the 5x-egfp Fragment

Based on our previous experiments, a linker consisting of five identical amino acids was designed and inserted between the Tat signal peptide and the N-terminal of *egfp* gene to generate a *5x-egfp* fusion gene fragment, where *x* here stands for a certain amino acid codon. Such *5x-egfp* fragment is found to be helpful for secretion from the bacterial host (data not shown). Since 5N-, 5Q- and 5R-EGFP were previously expressed in *E. coli* DH5α host, in order to construct the other 17

Table 19.1 Strain and plasmids used in this work

Bacterial strain and plasmids	Relevant properties	References
E. coli DH5α	–	Laboratory storage
pMD19-T simple vector	Amp^r	TaKaRa Biotechnology (Dalian) Co., Ltd.
pBT2	Amp^r Cm^r	A gift from University of Tuebingen, Germany
pBT2-Peftu-Tat-EGFP [8] (pBT2-ET-EGFP)	Amp^r Cm^r	Laboratory storage

kinds of *5x-egfp* fragment, the primers (Table 19.2) were correspondingly designed according to the sequence of *egfp* gene (No. AF302837).

The primers above were used to, respectively, amplificate the *5x-egfp* fragment using plasmid pBT2-ET-EGFP as PCR template. The following reaction mixture was set up as: 17.5 μL ddH_2O, 2.5 μL parent plasmid, 2.5 μL each primer, 25 μL 2× Taq PCR Master Mix. The cycling parameters for the PCR were as follows: 30 cycles of 95 °C for 5 min, 95 °C for 50 s, 70 °C for 30 s, 72 °C for 1 min followed by a single cycle of 72 °C for 10 min. The PCR products were purified by GeneJET Gel Extraction Kit (Thermo, Germany) after 1 % agarose gel electrophoresis.

19.2.3 Transformation of E. coli DH5α

The *E. coli* DH5α competent cells were thawed in ice for 5 min, then a 10-μL ligation product was added to the cells, and the mixture was incubated in ice for 30 min, heat-shocked for 90 s in a 42 °C water bath, immediately transferred to an ice bath for 1–2 min, followed by the addition of 1 mL sterile LB broth and constant shaking at 37 °C and 200 r/min for 1.5 h; a 100-μL culture broth was spread on LB agar plates containing 100 μg/mL chloramphenicol, and incubated at 37 °C for 12 h [9].

19.2.4 Preparation of Plasmid pBT2-ET-5X-EGFP

The resulting PCR products were, respectively, cloned into pMD19-T Simple Vector after overnight ligation at 16 °C and transformed into *E. coli* DH5α host. Plasmid pMD19-T-5X-EGFP prepared from *E. coli* was purified using the TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China). The constructed *5x-egfp* fragment was first digested by double digestion of *Hin*dIII and *Nhe*I (Fermentas, Lietuvos) from plasmid pMD19-T-5X-EGFP, then inserted into the synonymous sites in pBT2-Peftu-Tat-EGFP, a shuttle vector of *E. coli* and *Staphylococcus*, by T4 ligase (Fermentas, Lietuvos). After overnight ligation at 16 °C, the linked products were transformed into *E. coli* DH5α to create the recombinant plasmid

Table 19.2 Primers used in this work

Primer	Sequence	Underline sequence
5A-egfp-F	CAAGCTTGCAGCAGCTGCAGCAGTGAGCAAGGGCGAG	*Hin*dIII
5D-egfp-F	CAAGCTTGATGATGATGATGATGTGAGCAAGGGCGAGGAGCTGTTCACC	*Hin*dIII
5I-egfp-F	CAAGCTTATTATAATTATTATTGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG	*Hin*dIII
5G-egfp-F	CAAGCTTGGCGGAGGAGGAGGAGTGAGCAAGGGCGAG	*Hin*dIII
5N-egfp-F	CAAGCTTAATAACAATAATAACGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG	*Hin*dIII
5L-egfp-F	CAAGCTTCTTCTGTTACTTCTTGTGAGCAAGGGCGAGGAGCTGTTCACC	*Hin*dIII
5F-egfp-F	CAAGCTTTTTTTTTTCTTTTTTGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG	*Hin*dIII
5P-egfp-F	CAAGCTTCCTCCTCCGCCTCCTGTGAGCAAGGGCGAG	*Hin*dIII
5S-egfp-F	CAAGCTTTCATCAAGTTCATCAGTGAGCAAGGGCGAGGAGCTGTTCACC	*Hin*dIII
5T-egfp-F	CAAGCTTACAACAACAACAACAGTGAGCAAGGGCGAGGAGCTGTTCACC	*Hin*dIII
5W-egfp-F	CAAGCTTTGGTGGTGGTGGTGGGTGAGCAAGGGCGAG	*Hin*dIII
5Y-egfp-F	CAAGCTTTATTATTATTATTATGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG	*Hin*dIII
5V-egfp-F	CAAGCTTGTAGTAGTGGTAGTAGTGAGCAAGGGCGAGGAGCTGTTCACC	*Hin*dIII
5H-egfp-F	CAAGCTTCATCATCATCATCATGTGAGCAAGGGCGAGGAGCTGTTCAC	*Hin*dIII
5M-egfp-F	CAAGCTTATGATGATGATGATGGTGAGCAAGGGCGAGGAGCTGTTCAC	*Hin*dIII
5C-egfp-F	CAAGCTTTGTTGTTGTTGTTGTGTGAGCAAGGGCGAGGAGCTGTTCAC	*Hin*dIII
5K-egfp-F	CAAGCTTAAGAAAAAAAAAGAAGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGT	*Hin*dIII
5X-egfp-R	GGCTAGCGAATTCATTACTTGTACAGCTCGTCCATGCCGAGAGTGATCC	*Nhe*I

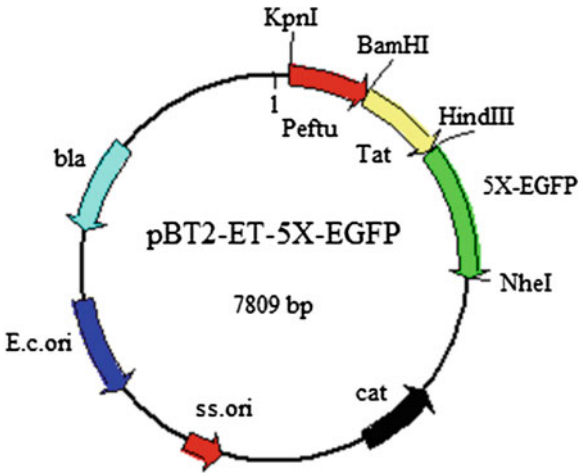

Fig. 19.1 Diagram of plasmid pBT2-ET-5X-EGFP. 5X-EGFP, EGFP with a 5 identical amino acids linker at its N-terminal (X represents for an amino acid of A, C, D, F, G, H, I, K, L, M, N, P, S, T, V, W and Y, respectively); *Tat* a twin-arginine translocation signal peptide of *efeB* gene in *S. carnosus* TM300; *Peftu* a strong promoter of *tufA* gene in *S. carnosus* TM300; *bla* ampicillin resistance gene; *cat* chloramphenicol resistance gene; *E.c.ori* origin of replication in *E. coli*; *ss.ori* origin of replication in *Staphylococcus*

pBT2-ET-5X-EGFP (Fig. 19.1). All plasmid constructions were verified by double restriction enzyme digestion and DNA sequence analysis.

19.2.5 Extraction of EGFP

Single colonies of *E. coli* DH5α/pBT2-ET-5X-EGFP and *E. coli* DH5α were, respectively, grown in 50 mL LB broth at 37 °C and 180 r/min for 16 h. The cell pellets were harvested by centrifugation at 12,000 r/min and 4 °C for 15 min. Proteins present in the culture medium were treated with 10 % trichloroacetic acid (TCA) for 12 h at 4 °C. The cells were resuspended in 2 mL PBS buffer, disrupted by sonication, and centrifuged to separate the supernatant from cell fragment. The supernatant was carefully transferred to a new vial and then precipitated by adding 10 % TCA for 12 h at 4 °C. The precipitated proteins were washed successively in 100 and 80 % acetone and dried at room temperature.

19.2.6 Detection of EGFP

After fermentation culture, the expression of the modified 5X-EGFP in *E. coli* DH5α host was observed by fluorescence microscopy (Olympus (China) Co., Ltd., China), the number of fluorescent cells per 10,000 was counted by flow cytometry

(Accuri C6, BD, America), and the expression of EGFP in cells and culture medium of *E. coli* DH5α/pBT2-ET-5X-EGFP was also analyzed by SDS-PAGE.

19.3 Result

19.3.1 Construction of 5x-egfp Fragment

The 17 kinds of *5x-egfp* fragment obtained from PCR were examined by agarose gel electrophoresis (Fig. 19.2a, b) and purified with GeneJET Gel Extraction Kit (Fig. 19.2c). Double restriction digestion and sequence alignment of plasmid pMD19-T-5X-EGFP (Fig. 19.2d) showed the same result with the target gene.

19.3.2 Verification of pBT2-ET-5X-EGFP

Recombinant plasmid pBT2-ET-5X-EGFP extracted from recombinant strain was examined by 1 % agarose gel electrophoresis (Fig. 19.3). Their molecular weights were expected as 8,000 bp for pBT2-ET-5X-EGFP and 800 bp for 5X-EGFP.

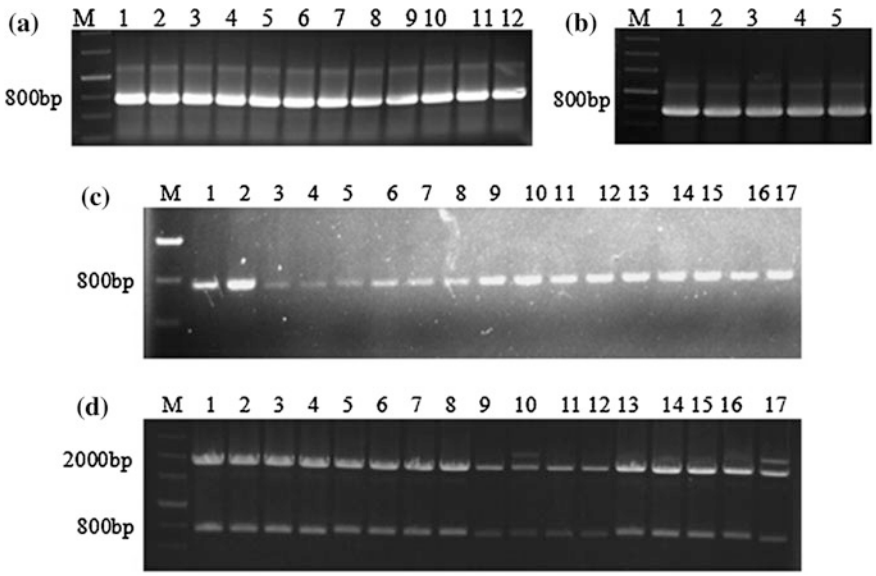

Fig. 19.2 Agarose gel electrophoresis of the PCR product of *5x-egfp* fragment. *M* DNA Marker; *a1–a12* PCR product of *5x-egfp* (x = codon for A, C, D, F, G, H, I, K, L, M, N or P); *b1–b5* PCR product of *5x-egfp* (x = codon for S, T, V, W and Y); *c1–c17* purified product of *5x-egfp* (x = codon for A, C, D, F, G, H, I, K, L, M, N, P, S, T, V, W or Y); *d1–d17* pMD19-T-5X-EGFP digested by *Hin*dIII and *Nhe*I (X = A, C, D, F, G, H, I, K, L, M, N, P, R, S, T, V, W or Y)

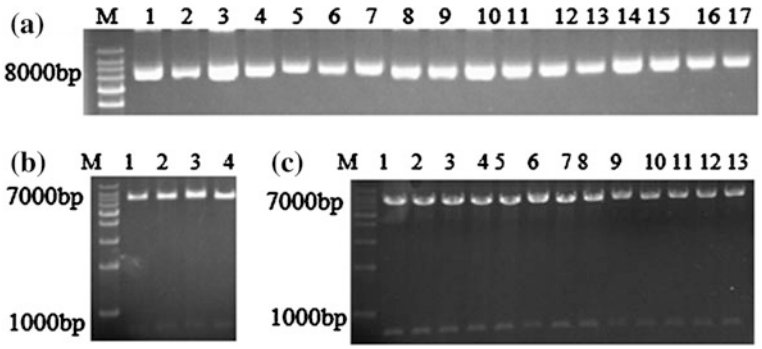

Fig. 19.3 Agarose gel electrophoresis of pBT2-Peftu-Tat-5X-EGFP. *M* DNA Marker. *a1–a17* pBT2-ET-5X-EGFP (X = A, C, D, F, G, H, I, K, L, M, N, P, S, T, V, W or Y); *b1–b4* pBT2-ET-5X-EGFP digested by *Hin*dIII and *Nhe*I (X = A, C, D or F); *c1–c13* pBT2-ET-5X-EGFP restricted with *Hin*dIII and *Nhe*I (X = F, G, H, I, K, L, M, N, P, R, S, T, V, W or Y)

19.3.3 Florescence Detection of EGFP in E. coli DH5α

A 10 μL culture broth of *E. coli* DH5α/pBT2-ET-5X-EGFP was used for florescence microscope observation. The *E. coli* DH5α host strain bearing pBT2-ET-5X-EGFP exhibited strong fluorescence, while the control strain showed no fluorescence (Fig. 19.4).

19.3.4 Detection of EGFP in E. coli DH5α by SDS-PAGE

Dried precipitated proteins in the culture medium and the supernatant were resuspended in 50 μL PBS. After adding 12.5 μL loading buffer, the 30 μL samples were

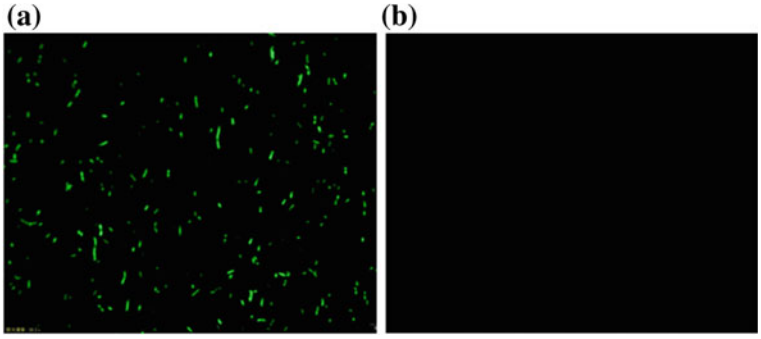

Fig. 19.4 Observation of *E. coli* DH5α/pBT2-ET-5X-EGFP and *E. coli* DH5α by fluorescence microscope. **a** *E. coli* DH5α/pBT2-ET-5X-EGFP; **b** *E. coli* DH5α

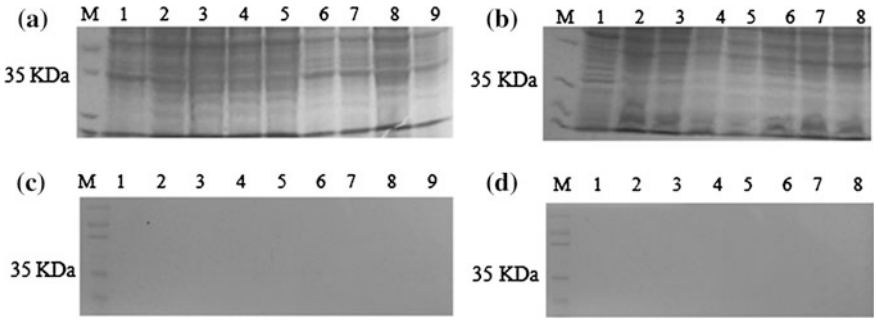

Fig. 19.5 Expression of 5X-EGFP in *E. coli* DH5α validated by SDS-PAGE. *M* Protein Marker (Fermentas, Lietuvos); *a1–a9* 5X-EGFP extracted from the cytoplasm of *E. coli* DH5α/pBT2-ET-5X-EGFP (X = A, C, D, F, G, H, I, K or L); *b1–b8* 5X-EGFP extracted from the cytoplasm of *E. coli* DH5α/pBT2-ET-5X-EGFP (X = M, N, P, S, T, V, W or Y); *c1–c9* 5X-EGFP extracted from the culture medium of *E. coli* DH5α/pBT2-ET-5X-EGFP (X = A, C, D, F, G, H, I, K or L); *d1–d8* 5X-EGFP extracted from the culture medium of *E. coli* DH5α/pBT2-ET-5X-EGFP (X = M, N, P, R, S, T, V, W or Y)

applied for SDS-PAGE analysis. The molecular weights of the 17 homologous target proteins were expected at about 35 kDa for 5X-EGFP. The results proved that 5X-EGFP were properly expressed in a soluble form in *E. coli*, but could not be translocated outside the cell wall (Fig. 19.5).

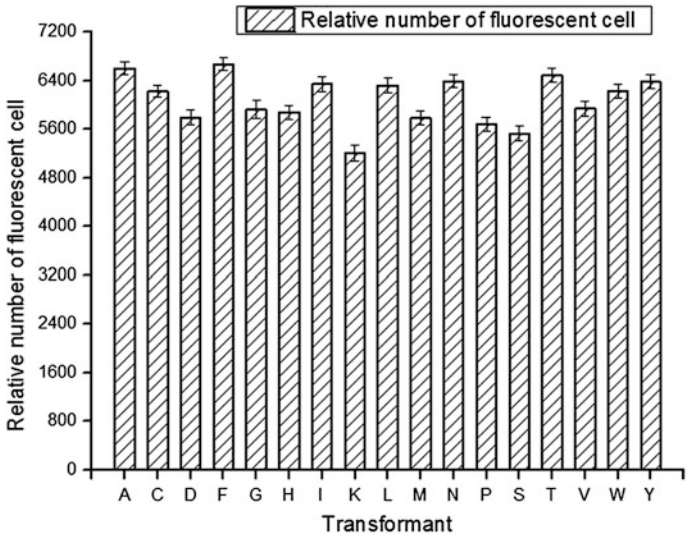

Fig. 19.6 Fluorescence of *E. coli* DH5α/pBT2-ET-5X-EGFP measured by flow cytometry. *X axis* transformants *A* to *Y* strand for *E. coli* DH5α/pBT2-ET-5X-EGFP; *Y axis* number of fluorescent cell per 10,000 cells

19.3.5 Expression of EGFP in E. coli DH5α Detected by Flow Cytometry

A 1-mL suspension of *E. coli* DH5α/pBT2-ET-5X-EGFP was analyzed by flow cytometry. An average of fluorescent cell number indicated that more than half of the cells expressed active 5X-EGFP in *E. coli* DH5α cells (Fig. 19.6).

19.4 Discussion

Tat pathway first found in *E. coli* was now a focus of protein transportation research around the world due to its feature to help translocate fully folded proteins across the bacterial plasma membrane. Bolhuis et al. [10] found that two integral cytoplasmic membrane proteins TatB and TatC make up a structural and functional unit of the twin-arginine translocases in *E. coli.* Tat-dependent heterologous protein secretion was analyzed in the three different Gram-positive bacteria *Staphylococcus carnosus*, *Bacillus subtilis,* and *Corynebacterium glutamicum* using GFP as reporter [11]. Differences about the final localization and the folding status of the exported GFP proved that the choice of potential bacteria host and suitable microorganism is essential in GFP secretion via Tat pathway.

In this study, the constructed 5X-EGFP was used as a model protein reporter to study the possibility of Tat-dependent heterologous protein secretion in *E. coli* DH5α. By means of SDS-PAGE, fluorescence microscope observation and flow analyzation, all the 17 kinds of 5X-EGFP were successfully expressed in a soluble and active form in *E. coli* DH5α cytoplasm, but failed to be translocated out of the cell wall. One possible explanation is the lack of a series of cofactor-binding-proficient proteins which plays an important role in protein transportation [12], the existence of Gram-negative *E. coli* outer membrane is another reason for this failure.

This research constructed an *E. coli-Staphylococcus* shuttle vector to study the function of Tat-pathway upon exogenous protein secretion. In the future research, the recombinant plasmid pBT2-ET-5X-EGFP is to be transformed into *Staphylococcus carnosus* TM300, a valuable genetic engineering strain in food industry [13] with low extracellular proteolytic activity and no by-products like toxin, hemolysin, coagulase [14], and thereby lays the preliminary experimental basis for further insight into heterologous protein secretion via twin-arginine translocation pathway.

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