CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals and equipments used in this study were shown in Appendix A.

2.2 Construction of phagemid containing Tat-CD147Ex gene

2.2.1 Site-directed mutagenesis of pComb8-CD147Ex phagemid

Site-directed mutagenesis was applied to make *Nsi* I restriction site at upstream of PelB signal sequence in pComb8-CD147Ex phagemid by using primers, MutPelBa (5'-GAG ACA GTC ATA atg cat TAC CTA TTG CCT ACG-3') and MutPelBb (5'-CGT AGG CAA TAG GTA atg cat TAT GAC TGT CTC-3'), in which the *Nsi* I restriction site is designated in small letters. Primers MutPelBa and MutPelBb (125 ng each) together with 50 ng of pComb8-CD147Ex and 2.5 U of PfuTurbo DNA polymerase (Stratagene, CA, USA) were resuspended in a 50 µl of PCR mixture. The PCR cycling condition was one cycle at 95 °C for 30 s, followed by 11 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68°C for 8 min. Subsequently, the mixture was treated with 10 U of *Dpn* I restriction enzyme (Promega, WI, USA) and incubated at 37 °C for 1 hr to digest the non-mutated parental vector. The resulting phagemid was named MpComb8-CD147Ex. The MpComb8-CD147Ex was digested with *Nsi* I and *Xba* I (Fermentas, MA, USA) to prepare the cloning site. After

purification, the 5'-end dephosphorylation was performed to prevent recircularization and religation of linear-digested MpComb8-CD147EX by using 0.04 U of calf intestinal alkaline phosphatase (Promega).

2.2.2 TorA signal sequences amplification by PCR

Two oligonucleotides, TatNsiIFw (5'-GAG GAG GAG GTa tgc atA ATA ACG ATC TCT TTC AG-3') with *Nsi* I restriction site (small letters) and TatXhoIRev (5'-GAG GAG GAG CTc tcg agC GCC GCT TGC GCC GCA GT-3') with *Xho* I restriction site (small letters) were used as primers for amplifying the TorA signal sequence gene from pSPL04 vector (kindly provided by Dr. J.H. Weiner, University of Alberta, Canada). 100 ng of pSPL04 vector was annealed with 250 ng of each primer in the 100 µl of the PCR mixture containing 5U of ProofStart DNA polymerase (Qiagen, Hilden, Germany). The amplification condition included a jump start at 95 °C for 5 min and followed by the three cycles of PCR amplification: denaturation at 94 °C for 50 s, annealing at 50 °C for 50 s and extension at 72 °C for 1 min. After 35 amplification cycles, the mixture was incubated at 72 °C for 10 min. The resulting 126 bp PCR product was analyzed by gel electrophoresis and subsequently treated with *Nsi* I and *Xho* I (Fermentas) at 37 PP^{o}C for 16 h and purified by QIAquick PCR purification kit (Qiagen).

2.2.3 Purification of PCR product by QIAquick PCR Purification Kit

Five volume of PB buffer was mixed together with 1 volume of PCR product and transferred to a QIAquick spin column, which was placed on a 2-ml collection tube. To bind the DNA, the QIAquick column was subsequently centrifuged at 10,000 g for 60 sec and discarded the flow through. Then, the

QIAauick spin column was washed by 0.75 ml of PE buffer and centrifuged 2 times for eliminating the flow through solution at 10,000 g for 60 sec. Finally, the DNA-binding column was replaced in a clean 1.5 ml microcentrifuge tube and eluted by loading 30 ml of distilled water and centrifuged at 10,000 g for 1 min. The size of DNA was checked by fractionating in 1% gel electrophoresis.

2.2.4 Construction of phagemid containing Tat-CD147Ex gene

The pComb8-CD147Ex phagemid was digested with *Xho* I and *Xba* I at 37 ^{o}C for 16 h. The DNA fragment of digested pComb8-CD147Ex containing CD147 gene, 844 bp was further purified by using QIAquick Gel extraction kit (Qiagen) and ligated with digested TorA signal sequence PCR product by using 5 U of T4 ligase enzyme (Roche Molecular Biochemicals, Mannheim, Germany). The 970 bp ligation product was named Tat-CD147 fragment. The Tat-CD147 ligated product was further amplified using primers, TatNsiIFW and Tat-CD147Rv (5'-GAG GAG GAG CTt cta gaA CTG ACG AGC TC-3', the *Xba* I restriction was designated in small letters). The PCR cycling condition was similar to which used for TorA signal sequence amplification. After purification, the PCR product was digested with *Nsi* I and *Xba* I at 37 ^{o}C for 16 h.

The 250 ng of *Nsi* I and *Xba* I digested Tat-CD147 fragment was ligated with 247 ng of dephosphorylated *Nsi* I and *Xba* I digested MpComb8-CD147Ex by using 5 U of T4 ligase enzyme (Fermentas) and the reaction mixture was incubated at 4 ^{o}C for 16 h. The newly constructed phagemid was named pTat8-CD147. The schematic diagram of pTat8-CD147 phagemid construction is shown in Figure 2.1

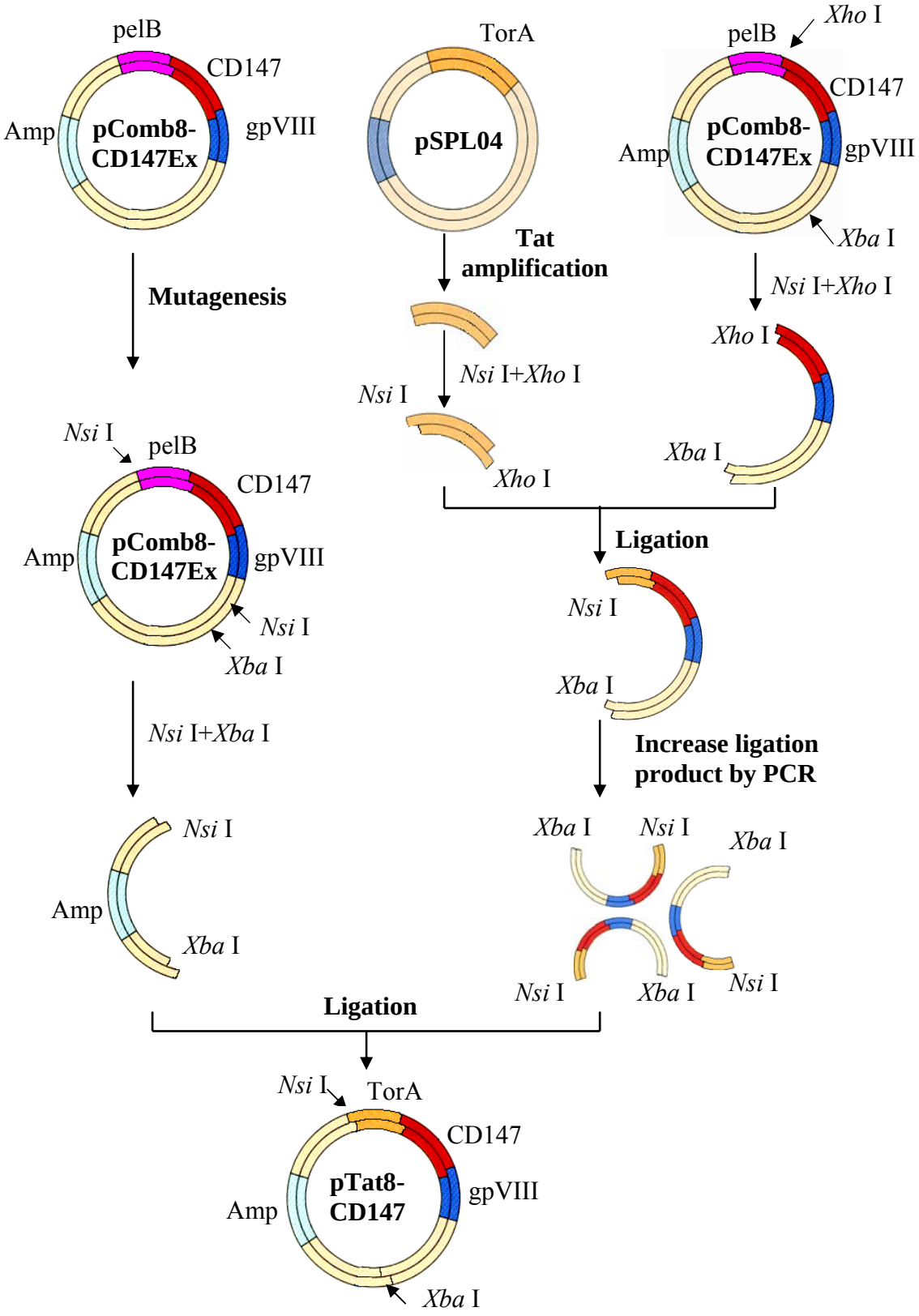

Figure 2.1 Schematic diagram of pTat8-CD147 phagemid construction.

2.3 Bacterial cell transformation

The ligation product was transformed into competent TG-1 wild type and competent Δ*tatABC* mutant strain (DSS640) (a gift from Dr. J.H. Weiner, Canada). The ligated DNA was co-incubated with 200 μl of cold-thawed $CaCl_2$ competent cells on ice for 1 h. The mixture was transferred into cooled screw cap tube and subsequently shocked at 42 °C for 1.5 min, then abruptly chilled on ice for 1 min. Three milliliter of non-antibiotic LB broth was added and further cultured with shaking (120 rpm) at 37 °C for 3 h. The transformed cell was centrifuged (2,500 rpm) at RT for 10 min and plated on ampicillin-containing LB agar (100 μg/ml). The plates were then incubated at 37 °C overnight (14-16 h)

2.4 Purification of phagemid by using alkaline lysis method

An ampicillin resistant colony was picked and grown in 3 ml of ampicillin containing LB broth (100 μg/ml) with vigorous shaking (180 rpm) at 37 °C for 8 h. The 1.5 ml of culture was centrifuged 10,000 g at 4 °C for 5 min. The supernatant was discarded and the cell wall of bacterial pellet was lysed by 100 μl of 1X glucomix-lysozyme and vortexed vigorously. Two hundred microliter of freshly prepared NaOH/SDS was added and mixed by inverting. Then, 150 μl of potassium acetate was added and gently mixed by vortex. The solution was centrifuged 10,000 g at 4 °C for 5 min for collecting the clear supernatant. The nine hundred microliter of analytical grade absolute ethanol was added and kept on ice for 2 min. The DNA was spun down at 10,000 g at 4 °C for 5 min and the supernatant was discarded. The DNA pellet was reconstituted by 100 μl of sterile DW and followed by adding 50 μl of

7.5M ammonium acetate and incubated at –70 °C for 10 min. The supernatant was collected by centrifugation at 10,000 g at 4 °C for 5 min. Three hundred microliter of absolute ethanol was added to the saved supernatant and incubated at –70 °C for 10 min. The solution was spun down to harvest the pellet. The pellet was cleaned up with 1 ml of 70% ethanol by centrifugation 10,000 g at 4 °C for 5 min. The DNA pellet was dried at 37 °C about 30 min and reconstituted with 30 µl of sterile DW and stored at –20 °C.

2.5 Characterization of recombinant clones

The purified phagemids were firstly checked by fractionating in 1% agarose gel electrophoresis. In order to verify the correct *E. coli* clones, the purified phagemid from the individual clone was characterized by digesting with *Nsi* I, *Xho* I, *Spe* I and *Xba* I to identify the band of correct insert. The PCR reamplification was used to determine the correct size of Tat-CD147 fragment.

2.6 Phage-displaying CD147Ex *via* gpVIII

2.6.1 Phage-displayed CD147ExgpVIII preparation

After transforming TG-1 wild type and DSS640 strain with pTat8-CD147, the phage display technique was performed by using the phage displaying CD147 by Sec pathway protocol as described previously (Intasai *et al.*, 2003). Briefly, a clone of pTat8-CD147 transformed TG-1 was grown in 10 ml of 2×TY broth (1.6% [w/v] tryptone, 1% [w/v] yeast extract, and 0.5% [w/v] sodium chloride) containing 100 µg/ml ampicillin at 37 °C until an optical density (OD) at 600 nm of 0.8 was

reached. The precultured bacteria were subsequently propagated in 100 ml of the same medium containing 2 ml of 50% glucose and cultured at 25 ^{o}C until reaching 0.5 OD. The 30 ml of culture was infected with 250 µl of 10^{12} pfu/ml of the VCSM13 helper phage and left at 37 °C for 30 min without shaking. Phage-infected TG-1 was spun down at 3000 rpm for 10 min at 4 °C. The pellet was resuspended in 30 ml 2×TY broth containing ampicillin (100 µg/ml) and kanamycin (70 µg/ml) and then transferred to 220 ml of the same broth and shaken at 180 rpm for 16 h at 25 °C.

2.6.2 Harvesting phage by PEG precipitation

Bacteriophages harboring CD147Ex *via* gpVIII were centrifuged 3,300 rpm at 4 °C for 30 min. The culture supernatant was collected and phages were precipitated by 4% w/v of PEG 8,000 and 3% w/v of NaCl with shaking (180 rpm) for 15 min at RT or until PEG and NaCl were completely dissolved. The supernatant was kept on ice for 30 min and centrifuged 10,800 rpm at 4 °C for 30 min. The pellet was air dried for 30 min and reconstituted in 2.5 ml of 1 mM PBS pH 7.2. The solution was centrifuged 12,000 rpm at 4 °C for 10 min and the supernatant was preserved in 30% glycerol. The precipitated phage was stored at –70 °C.

2.6.3 Phage titration by reinfecting the *E. coli* cell

The recombinant phages were further titrated by reinfecting into *E. coli* strain TG-1. One microliter of precipitated phage was transferred to 999 µl of 1 mM PBS to make a dilution of 1:10^3, then 1 µl of the mixture was used to infect 1 ml of cultured *E. coli* TG-1 having OD600 at 0.5 (phage final dilution is 1: 10^6). Fifty

microliter of viral-infected bacteria was plated on LB agar containing 100 μg/ml of ampicillin. The final dilutions of phage infected TG-1 at 1: 10^8 and 1: 10^{10} were performed by the similar procedure. Plates were incubated at 37 °C overnight and the ampicillin resistant colonies were counted and calculated for phage concentration.

2.7 Detection of the phage-displayed CD147ExgpVIII by immunological techniques

2.7.1 Sandwich ELISA for phage-displayed CD147ExgpVIII

A microtiter plate (NUNC, Roskilde, Denmark) was coated with 50 μl of 10 μg/ml CD147 mAbs (M6-1E9; IgG_{2a}, M6-1D4; IgM, M6-1F3; IgM, M6-2B1; IgM and M6-2F9; IgM (Kasinrerk *et al*., 1999) in carbonate/bicarbonate buffer, pH 9.6 at 4 °C for 18 h. The phage-coated wells were blocked with 2% skimmed milk diluted in 1mM PBS at RT for 1 h. The wells were washed 4 times with washing solution (0.05% Tween 20 diluted in 1 mM PBS). Phages displaying CD147Ex *via* gpVIII by Tat pathway (ΦTat-CD147gpVIII) were added to the wells by using optimized phage amount (50μl of 10^{10}-10^{11} cfu/ml). After incubating at room temperature for 1 h, the plate was washed to remove unbound phages. Binding of phage particles was monitored by using two alternative sets of anti-M13 mAb. One was HRP-conjugated anti-gpVIII mAb (Amersham–Pharmacia Biotech, Buckinghamshire, UK). The other was biotinylated anti-gpIII mAb (Exalpha Biologicals, MA, USA). HRP-conjugated anti-biotin (Zymed, CA, USA) was applied to trace biotinylated anti-gpIII mAb. Following washing, the 3,3′,5,5′-tetramethylbenzidine substrate was added and incubated at room temperature for color

development. OD at 450 nm was determined after adding 1 N HCl to stop the reaction. Phages displayed CD147Ex *via* gpVIII by Sec pathway (ΦSec-CD147gpVIII), phages displayed CD147Ex *via* gpVIII which is prepared from TG-1 Δ*tatABC* mutant strain (ΦTatmut-CD147gpVIII) and VCSM13 helper phages were used as controls in the ELISA system.

2.7.2 SDS–PAGE and Western immunoblotting

Protein components of precipitated phages were separated by SDS-PAGE under reducing condition on 12% polyacrylamide gel. For Western immunoblotting, the separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane. Blotted membrane was blocked at 4 °C for 18 h in 5% skim milk in 0.15 M PBS, pH 7.2, and then incubated with the pooled CD147 mAbs (M6-1E9, and M6-1D4 at 5 μg/ml each) for 1 h at room temperature on a shaking platform. Following washing four times with 0.05% Tween 20 in 0.15 M PBS, pH 7.2, HRP-conjugated rabbit-anti-mouse immunoglobulins antibody (Zymed) diluted in 5% skim milk in 0.15 M PBS, pH 7.2, was added to the membrane and incubated at room temperature for 1 h. After washing step, the immuno-reactive bands were then visualized by chemiluminescent substrate detection system (Amersham–Pharmacia Biotech).