Site-Directed Mutagenesis Analysis of PilS, a Type IVB Pilin from *Salmonella typhi*

Lui E.Y.¹ and Swaminathan K.²

Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore

ABSTRACT

PilS is a Type IVb pilin and forms the pili of *Salmonella typhi* which is a major adhesion factor for this pathogen to enter the human gastrointestinal epithelial cells and cause typhoid fever in infected patients. The aim of this project is to investigate the impact of mutation of two positively charged amino acids (Lys-75 and Lys-120) of the PilS protein on binding to cystic fibrosis transmembrane conductance regulator (CFTR), which is the epithelial cell receptor of *S. typhi*. The project was started with cloning of the DNA fragment for *S. typhi* ΔPilS (residues 26-181) in the modified pET-32a vector. It was followed by site-directed mutagenesis of the two indicated amino acids to alanine. Several methods were tried in order to get the mutated ΔPilS DNA. The mutated ΔPilS DNA was finally obtained by using the overlap extension PCR method. However, transformation of this mutated ΔPilS DNA into competent cells failed and no colony was formed. Due to time constraint, further experiments for this project could not be carried out and should be followed by other researchers in the laboratory.

INTRODUCTION

Type IV pili are found on many bacterial species and play an important role in pathogenesis and host-cell adhesion (Forest & Tainer, 1997; Balakrisna et al., 2006). They are homopolymers of single chain pilin protein and are required for virulence in bacterial pathogens that cause diseases such as cholera, typhoid, pneumonia, gonorrhea and meningitis (Balakrisna et al., 2006). Type IVb pili have been identified on bacteria such as *Vibrio cholera*, *Salmonella typhi*, enteropathogenic *Escherichia coli* and the enterotoxigenic *E. coli* where they colonize in the human intestine. The type IV pilus of the bacteria *S. typhi* is believed to be a major adhesion factor for the bacteria to enter into gastrointestinal epithelial cells following oral intake (Balakrisna et al., 2006).

The type IVb pilus operon of *S. typhi* contains the PilS gene which encodes the a pilin protein (Tsui et al., 2003). The structure of 26-N-terminal-truncated type IVb structural pilin monomer (ΔPilS) was determined by using the NMR technique (Xu et al., 2004) and is under investigation using X-ray crystallography (Balakrisna et al., 2006). Studies showed that the target receptor on human intestinal cells for *S. typhi* pilus is a stretch of 15-mer peptide at the first extracellular domain and the binding site is at residues 113-117 (NKEER) of cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial cell receptor for the type IV pili prior to entry of the

¹ Undergraduate
² Supervisor
gastrointestinal submucosa (Tsui et al., 2003; Xu et al., 2004; Swaminathan, personal communication).

A model of *S. typhi* pilus has been suggested and mutagenesis studies showed that the residues on the αβ loop and the C-terminal disulphide-bonded region of PilS protein involved in the assembly and binding specificity of the pilus (Xu et al., 2004). Lys-75 and Lys-120 are located at the active site pocket of the pilin molecule on the α2-β1 loop and β4-α3 loop respectively (Xu et al., 2004; Swaminathan, personal communication). Interestingly, the CFTR peptide contains four negatively charged residues at peptide 108-117, thus it was expected that the binding site of the PilS protein to contain polar or positively charged residues for specific binding. Moreover, the crystallographic study of the ΔPilS-CFTR complex shows a conformational change of ΔPilS at Lys-75 and Lys-120, hence it is believed that these charged residues play a crucial role in the binding of the PilS protein with the receptor (Swaminathan, personal communication).

Therefore, the aim of this project is to investigate whether the mutation of the Lys-75 and Lys-120 charged residues into neutral residues would have an impact on the binding of PilS protein with the CFTR receptor. It is hypothesized that this mutation can prevent the PilS protein from binding to the CFTR peptide and hence can give the understanding on the binding architecture of PilS pilus to CFTR.

**MATERIALS AND METHODS**

**Cloning**

The starting material was the modified pET-32a (Novagen) vector (with the S-tag and thioredoxin gene removed) containing the *S. typhi* ΔPilS (residues 26-181) DNA fragment between the BamHI and EcoRI sites. The project started with transformation of this pET32a:ΔPilS into DH5α competent cells. After an overnight culture at 37 °C, single colonies were picked up and inoculated into LB broth containing 0.2% ampicillin and were grown overnight at 37 °C. The culture was then isolated using miniprep (GeneAll® Exprep™ Plasmid Quick kit). The concentration of the purified DNA was measured by nanodrops (GE Healthcare) and the correct sequence of the construct was confirmed by sequencing PCR.

**Site-Directed Mutagenesis**

The DNA was tried to be mutated at Lys75 and Lys120 by using the Invitrogen™ GeneTailor™ Site-Directed Mutagenesis system. Primers were designed according to the specifications provided in the protocol. It was then followed by a methylation reaction to methylate cytosine residues throughout the DNA and Standard reaction Setup using Platinum® Platinum® pfx DNA Polymerase. The product was analysed on a 1% agarose gel. Had this reaction succeed, the mutated DNA will be transformed into DH5α *E. coli* competent cells and *McrBC* endonuclease in the host cell would digest the methylated template DNA, leaving only unmethylated, mutated product. The whole process was repeated by adding in 5% DMSO after no band was observed for the previous trials. The product was analysed on a 1% agarose gel together with a positive control.

**Site-Directed Mutagenesis Using Overlap Extension PCR**

The mutagenesis reaction was repeated by using an established protocol of site-directed mutagenesis. Primers were designed to clone the 5’ segment (F1 and R1), the middle segment
(F2 and R2) and the 3’ segment (F3 and R3) of PilS DNA. Mutagenesis PCR was run using these three pairs of primers. The three DNA constructed were then combined by gradient PCR using F1 and R3 primers only to create the full length of mutated ∆PilS DNA. The combined DNA construct was then amplified by using PCR. The products were confirmed on a 1% agarose gel. It was then followed by gel extraction using the GeneAll® ExpinTM Gel SV protocol. The concentration of the mutated DNA was measured.

Double Digestion
The modified pET-32a vector and the DNA product were each double digested with BamHI and EcoRI restriction enzymes in a 37 °C water bath for two and a half hours. The products were separated from the digested ends by running on a 1% agarose gel and followed by gel extraction using the GeneAll® ExpinTM Gel SV protocol.

Ligation
The double digested vector and DNA were ligated at the DNA to vector ratio of 3:1 and 10:1 at room temperature for two hours. It was followed by transformation into BL21 (DE3) strain of E. coli competent cells to get construct transformed colonies.

RESULTS AND DISCUSSIONS

Human typhoid fever is an infection caused by the bacterium *Salmonella typhi*. They enter the enterocytes and specialized M cells of the small intestine for submucosal translocation (Jones, 1996). It is suspected that two charged residues on PilS (Lys-75 and Lys120) are responsible for the binding of the PilS pilus to the CFTR peptide and aid in entry and translocation of *S. typhi* into the intestinal submucosa.

Cloning
The transformation of the modified pET-32a vector containing the *S. typhi* ∆PilS into DH5α competent cells was successful and colonies were formed. After isolation of the DNA from the culture by miniprep, the sequencing PCR reaction confirmed that the construct was correct.

Site-Directed Mutagenesis
The mutagenesis using the Invitrogen™ GeneTailor™ Site-Directed Mutagenesis kit did not yield any band on 1% agarose gel. It was suspected that it was because the Platinum® pfx DNA polymerase was ineffective. This step was repeated using a new Platinum® pfx polymerase but it did not give any band on agarose gel as well. Hence, it was suspected that the primers were forming primer-dimers which rapidly consume dNTPs and primers in the solution. The experiment was repeated by adding 5% DMSO to facilitate DNA strand separation and this reaction yielded a very faint band and it was believed that it was not the DNA that we wanted. The experiment was repeated again to include a positive control as well, but again it did not yield any band on agarose gel. It was concluded that the kit does not work for this DNA. The project proceeded to another method to generate the site-directed mutagenesis.

Site-Directed Mutagenesis Using Overlap Extension PCR
For this method, primers were designed such that Lys-75 and Lys-120 would create a mutation to become alanine residues and there will be overlaps for the different segments
created for subsequent combination. In the first round of PCR, primers F1-R1, F2-R2, F3-R3 were used to amplify the regions of interest as well as to introduce the site-directed mutation. In the second round of PCR, these amplified products were used as a megaprimer to amplify the entire region of interest together with the mutation introduced (Aiyar et al., 1996). The mutagenesis reaction using this method yielded bands on agarose gel which indicated that the intended segments of DNA was formed using a different pairs of primers. Positive control was included to ensure that the PCR reactions worked.

The DNA products were combined by gradient PCR using the F1-R3 primer pair to create the full length of DNA. Due to time constraint, this step was carried out using different annealing temperature at once (gradient PCR) and it was found that annealing temperature at 51.2 °C gave the best result.

**Double Digestion And Ligation**

The vector and DNA were double digested separated with the BamHI and EcoRI restriction enzymes. Gel extraction was performed and the concentrations of the DNA and vector from gel extraction were not high, but it was enough to carry out the next steps. The pET32a vector and DNA were ligated and transformed into *E. coli* competent cells. However no colony was formed. This step was repeated for several times but it did not seem to be successful.
FUTURE WORK

Due to time constraint further experiments could not be continued and should be followed by other researchers in the laboratory. Once the mutated ΔPilS can be ligated into a vector, vector:ΔPilS can be transformed and the mutant can be expressed. After purification, X-ray crystallography analysis could be carried out to investigate the change of binding ability of the mutate ΔPilS pilin with CFTR peptide.

By understanding the subunit structure and assembly architecture of the PilS-CFTR interaction, we can learn about pilus functions. This will help in the development of suitable vaccines and therapeutics to block the invasion of *S. typhi* into the epithelial cells of the intestine.

REFERENCES


