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CLONING, EXPRESSION AND PURIFICATION OF AcrV TIP PROTEIN FROM *Aeromonas hydrophila* USING *Escherichia coli* HOST CELLS

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ABSTRACT

Aeromonas spp. uses T3SS to secrete and transport effector proteins to the host cells. These proteins play a major role in bacteria virulence by interfering with the signaling cascades and disrupting the cytoskeleton structure of the host cell. Despite tremendous efforts, structural and functional information regarding AcrV tip protein of T3SS remains elusive. In this study, we cloned the gene encoding the AcrV protein from *Aeromonas hydrophila* AH-1 and inserted it into the pET-M expression vector. The pET-M vector containing AcrV gene was transformed and expressed in *E.coli* BL21 (DE3) cells. The recombinant AcrV protein was purified by affinity chromatography using Ni-NTA column. The obtained AcrV with high purity can be used for structural and functional studies.

Keywords: AcrV, Aeromonas hydrophila, affinity chromatography, gene expression, recombinant protein.

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INTRODUCTION

Aeromonas hydrophila is identified as a pathogen of fishes, reptiles, and amphibians (Shotts et al., 1972). During the last decade, *A. hydrophila* has gained its fame as more *Aeromonas* strains were found to be associated with a wide range of diseases in humans. Initially, *Aeromonas* strains were thought to be opportunistic pathogens feasting on immuno-compromised patients. They are now known to be the cause of septicemia and many other gastrointestinal diseases in humans (Fiorentini et al., 1998; Thune et al., 1993). Type III secretion system (T3SS) is essential for *A. hydrophila* pathogenesis. By using T3SS, *A. hydrophila* can inject its effector proteins into the host-cell (Yu et al., 2004). The three main components of T3SS are base, needle (composed of needle filament and tip complex), and translocon. Until now, the structure and function of proteins in T3SS have been considered and studied. However, the structure of the tip-complex remains elusive. It is crucial to determine the pathogenesis mechanism of bacteria, which will help develop effective drugs.

The tip complex which caps the needle filament plays a role in detecting host cells and sensing the environment (Epler et al., 2012). In the V-tip family protein (AcrV, PcrV, and LcrV), the structure of AcrV is still not available to date which limits our understanding about the function of this important protein. In *Yersinia* species, the assembly of translocator YopB-YopD pore is mediated by LcrV (Goure et al, 2005; Pettersson et al., 1999). The LcrV tip complex is predicted to be a pentameric ring, with LcrV protein being composed of N-terminal globular domain, the coiled-coil region, and C-terminal globular domain (Lara-Tejero and Galan, 2019). In 2005, Mueller et al. showed that *Aeromonas salmonicida* and *P. aeruginosa* injectisome needles had a tip complex formed by PcrV and AcrV (Mueller et al., 2005). Finding the tip complex has clarified how the translocation pore is formed in the host cell membrane but the exact role of AcrV remains to be investigated. In this study, the AcrV gene was cloned from *Aeromonas hydrophila* AH-1 and expressed in *E.coli* BL21 (DE3) cells under the control of T7 promoter of pET-M expression vector. The recombinant AcrV protein was purified by affinity chromatography. The research provides recombinant AcrV proteins of high purity, which can be used for structural and functional studies.

MATERIALS AND METHODS

The gene encoding AcrV was codon-optimized based on the AcrV gene sequence of *A. hydrophila* AH-1 (Accession No: AY394563.2). The codon - optimized sequence was chemically synthesized by Phusa Biochem (Can Tho, Vietnam). The pET-M expression vector was a modified product from pET32a, with the S-tag and theorin tag removed. The *E.coli* DH5-α and *E.coli* BL21 DE3 were cloning and expression host cells, respectively. Chemicals in this experiment were purchased from international companies, including Bio-rad, Sigma, Merck, Thermo scientific, and Serva in the United States or Germany.

Construction of recombinant expression vector

The gene encoding AcrV obtained from Phusa Biochem was amplified by PCR using forward primers AcrV-F gcGGATCCGAAATTAGCTCGTATAAAA AAGATCC with *Bam*HI site and reverse primers AcrV-R cgGTCGACTTAAATCGCCTGCAGAATCT G with the *Sal*I cleavage site. The PCR reaction used Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer's recommendations.

The PCR product and pET-M plasmid were digested by *Sal*I and *Bam*HI Fastdigest. For ligation DNA and vector, T4 DNA ligase of Thermo scientific was used. The ligated construct was then transformed into *E.coli* DH5alpha competent cells. PCR screening method was used to confirm the proper insertion of the AcrV gene into the plasmid vector. Colonies that contained DNA inserts of the correct sizes from the PCR screen were picked and cultured in LB environment with

100 µg/ml of ampicillin overnight. Subsequently, plasmids were extracted from *E. coli* cells using QIAGEN's Miniprep Kit kit. Plasmid products were sent for sequence analysis using the Sanger method at 1st Base (Singapore).

Expression of recombinant AcrV

The pET-M-AcrV construct was transformed into *E.coli* BL21 (DE3). One single colony was then transferred into 10 ml LB containing 100 µg/ml of ampicillin. 5 ml of the overnight culture was inoculated into 1 L of LB containing 100 µg/ml of ampicillin and grown at 37 ºC with shaking until OD_{600} reached 0.6. IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the culture at a final concentration of 0.3 mM. Cells were further grown at 25 ºC for 16 hours before harvesting by centrifugation for 10 min at 6000 rpm. The supernatant was discarded and the cell pellets were stored at -20 ºC until purification.

Purification of recombinant AcrV using affinity chromatography

Cells pellets from 1 L of overnight culture were resuspended in 25 ml of binding buffer (Tris-HCl 50 mM, pH 8.0; NaCl 300 mM; imidazole 5 mM). Cells were lysed by sonication on ice with 40 % amplitude and for 6 rounds of 5 min each. The lysate was centrifuged for 30 min at 13000 rpm and 4 ºC. The supernatant was collected and loaded into Econo-column (Biorad) containing Ni-NTA bead pre-equilibrated with 20 ml of binding buffer. The column was subsequently washed 10 rounds with binding buffer and 10 rounds with washing buffer (Tris-HCl 50 mM, pH 8.0; NaCl 300 mM; 30 mM imidazole) to remove unbound proteins. The His-tagged proteins were eluted with 20 ml of Elution buffer (Tris-HCl 50 mM, pH 8.0; NaCl 300 mM; imidazole 400 mM). The eluted proteins were dialyzed overnight against 20 mM Tris-HCl pH 8.0, 200 mM NaCl and concentrated to 15 mg/ml, aliquoted into 1.5 eppendorf tubes, and stored at -80 ºC.

RESULTS AND DISCUSSION

Clonning of AcrV gene into pET-M expression vector

The AcrV gene was amplified using oligonucleotide primers containing *Bam*HI and *Sal*I restriction enzyme sites. The pUC-19-AcrV plasmid template for PCR was chemically synthesized by Phusa Biochem. PCR result was shown in Figure 1A. The AcrV lane is a PCR product with only one 1104 bp band corresponding to the size of AcrV gene. The AcrV PCR product was inserted into the pET-M expression vector. After screening, the plasmid pET-M-AcrV was extracted (Fig. 1 (B)) in lane 1, and then was sequenced by the Sanger method.

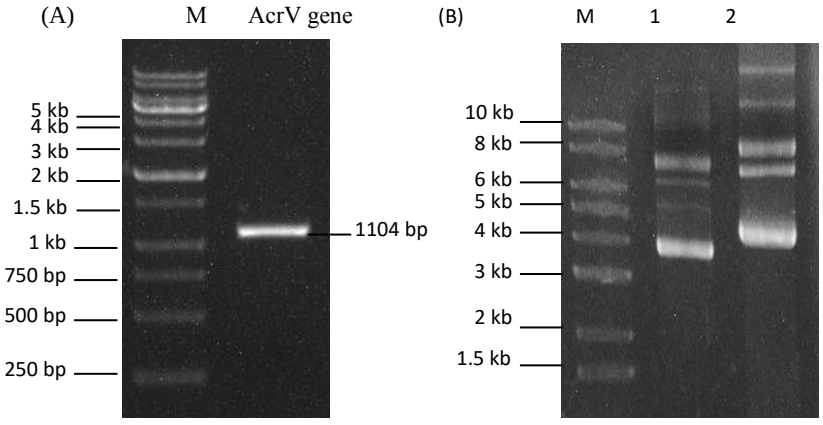

Figure 1. Cloning of AcrV gene into pET-M vector, (A) M: DNA marker 1kb; AcrV: AcrV gene was amplified from pUC-19-AcrV plasmid. (B) Result of plasmid extraction. (1): Plasmid pET-M, (2): Plasmid pET-M containing AcrV gene

Plasmid DNA sequencing

The sequencing result of pET-M AcrV was translated into the amino acid sequence using Snapgene and compared with the amino acid sequences of AcrV from *A. hydrophila* AH-1 published in the GenBank (Accession No: AY394563.2). The sequence comparison by CLUSTALW was presented in Fig. 2. These results indicated that the sequence was identical to the AcrV coding sequence. This confirms that we have succeeded in creating the expression vector carrying the AcrV gene.

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AcrV-Sequencing   MHHHHHHSSGLVPRGSMEISSYKKDPQLFLSDLGKVELNQLQGGSSSALDALVKLLQEKK  60
AcrV-genbank      ----------MEISSYKKDPQLFLSDLGKVELNQLQGGSSSALDALVKLLQEKK  44
                  ****************************************

AcrV-Sequencing   IVITATYDKKIDSNPFADKVVTENEMLLKKVLAYFMPADSKNSGGQYDLQIKAGFEQLHK  120
AcrV-genbank      IVITATYDKKIDSNPFADKVVTENEMLLKKVLAYFMPADSKNSGGQYDLQIKAGFEQLHK  104
                  ************************************************************

AcrV-Sequencing   LINEAAAAGKTKFTLREFLAATHFSLTPDRIDDDVIGAMLDAMGSHSSKRDTLKHEVGKL  180
AcrV-genbank      LINEAAAAGKTKFTLREFLAATHFSLTPDRIDDDVIGAMLDAMGSHSSKRDTLKHEVGKL  164
                  ************************************************************

AcrV-Sequencing   TAELRSYSIIQTEISTAQQHNGTVEVGRKGVNIFDYKHYGYDSHEAFAKKDANGQYNPQY  240
AcrV-genbank      TAELRSYSIIQTEISTAQQHNGTVEVGRKGVNIFDYKHYGYDSHEAFAKKDANGQYNPQY  224
                  ************************************************************

AcrV-Sequencing   QLLKEIAVERKETVLKEESLLRAEATDQGFSLEDYRTKLQTELVDLKNDKQVFLSARDFL  300
AcrV-genbank      QLLKEIAVERKETVLKEESLLRAEATDQGFSLEDYRTKLQTELVDLKNDKQVFLSARDFL  284
                  ************************************************************

AcrV-Sequencing   ISPKKDTGALSNVKYSYKYEKDNNPLSNFATTVGDRAKPLNDKLGQKTTELNDISSRYNA  360
AcrV-genbank      ISPKKDTGALSNVKYSYKYEKDNNPLSNFATTVGDRAKPLNDKLGQKTTELNDISSRYNA  344
                  ************************************************************

AcrV-Sequencing   VIEALNRFIQKYESVMQQILQAI* 383
AcrV-genbank      VIEALNRFIQKYESVMQQILQAI* 367
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Figure 2. Amino acid sequence alignment of recombinant AcrV gene

AcrV-Sequencing: the amino acid sequence of AcrV was inserted into pET-M; AcrV- GenBank: the amino acid sequence of AcrV (Accession No: AY394563.2) was published in GenBank.

Expression of AcrV in *E. coli* BL21 (DE3)

AcrV was over-expressed in *E. coli* BL21 (DE3) cells with 0.3 mM IPTG at 25 ^{o}C. The AcrV expression result was analyzed by SDS-PAGE electrophoresis (Figure 3). The results indicated that AcrV protein was only expressed with 42 kDa band in IPTG-induced *E. coli* BL21 (DE3) harboring pET-M - AcrV (Fig. 3, lane AcrV+IPTG), while this band did not show in the control sample.

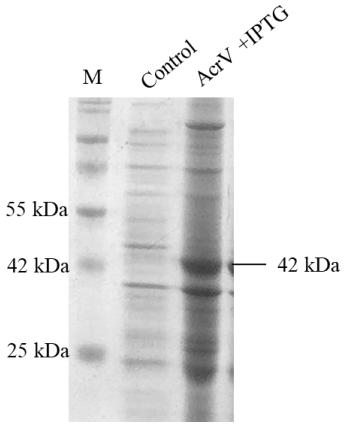

Figure 3. Expression of AcrV in *E. coli* DE3, M: Protein ladder; Control: non-induced cell; AcrV+IPTG: cells after IPTG induction

Purification of His-tag AcrV

AcrV was expressed as a soluble His-tag protein in *E. coli* BL21 (DE3) cells. Cells were lysed by sonication. Ni-NTA beads were used to separate the His-tag protein from other bacterial proteins. SDS-PAGE result in Figure 4 showed that protein 6xHis- tag AcrV was purified with a thick specific band at 42 kDa (lane 7 - Figure 4). The eluted protein was dialyzed against buffer containing 20 mM Tris-HCl pH 8.0, 200 mM NaCl to remove imidazole from the elution buffer. Approximately 50 mg of recombinant AcrV protein was obtained from one liter of LB culture. The purified protein was concentrated to 15 mg/ml, aliquoted into microcentrifuge tubes, and stored at -80 ºC.

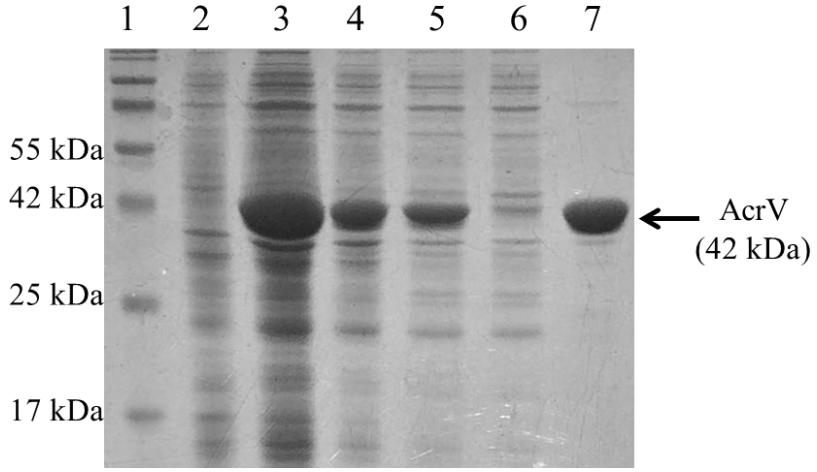

Figure 4. Purification of His-tag AcrV, 1: Protein ladder; 2: non-induced cell; 3: cell after IPTG induction; 4: cell pellet after sonication; 5: soluble protein after sonication; 6: flow-through from Ni-NTA affinity column; 7: eluted protein from Ni-NTA column

CONCLUSION

AcrV gene was cloned into pET-M vector and induced the expression in *E. coli* BL21 (DE3). Protein was expressed in LB medium at 25 ºC, induced by 0.3 mM IPTG when OD_{600} reached 0.6 and harvested after 16 hours of induction. The recombinant AcrV tagged 6x-His had a molecular weight of 42 kDa. Protein AcrV was purified with high purity by affinity chromatography with nickel bead. The obtained AcrV with high purity can be used for structural and functional studies.

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