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CLONING AND EXPRESSION OF PapG.AcmA FUSION PROTEIN IN *E. coli*

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) is one of the most common bacteria which can lead to urinary tract infection (UTI). The use of *Lactic acid* bacteria, as a delivery system, is one of the promising methods to develop the efficient vaccine. The aim of this study was the cloning and expression of PapG.AcmA fusion protein ~~on~~ in *E. coli*.

In this study, the PapG coding gene sequence fused to the AcmA gene (Anchor protein in *Lactobacillus*) was synthesized and cloned into the pEX-A vector and the fusion gene was sub-cloned into the pET21a expression vector. Subsequently, the recombinant plasmids (pET21a-PapG.AcmA) were transformed into the *E. coli* Origami strain and the fusion protein was expressed.

The results showed that the PapG.AcmA fusion was successfully sub-cloned in pET21a expression vector. The expression of PapG and PapG.AcmA proteins in the *E. coli* origami strain indicated a protein band in SDS-PAGE, confirmed by western blotting. Furthermore, the mass production and purification of PapG-AcmA protein with Ni-NTA column was done. Finally, the endotoxin analysis of the product was assayed by the limulous amoebocyte lysate test.

The results of the present study show that the PapG.AcmA fusion protein may be used as an effective target for vaccine production.

Keywords: PapG.AcmA, *E. coli*, expression

INTRODUCTION

Urinary tract infection (UTI) is the colonization and invasion of bacteria into the tissues of the urinary tract. The infection in humans is influenced by gender age and is common in young women [1-3]. UPEC bacteria often originate from feces and once microorganisms overcome the host's immune system, they will be able to colonize in the lower urinary tract and establish the infection [3-4]. The infection caused by UPEC is usually self-limiting and rarely spread beyond the urethra. However, UPEC bacteria invade to bladder epithelial cells by intracellular amplification, and upon exiting the cell's surface, invade to other epithelial cells in the tissues of the bladder, establishing a persistent infection [2, 5]. Due to urinary flow, mucus and secretory IgA secretion as well as bactericidal property of urine epithelial cells, urinary tract is normally sterile. However, some strains of E.coli bacteria can grow in the urinary tract because they share several virulence factors such as adhesion molecules and toxins [6] .The early step in the colonization of UPEC strains on the mucosal surfaces of the host is dependent on some adhesion molecules such as pili S, adhesion family of Dr, pili p and pili type I [7, 8]. Among them, type I and p Pili in the adhesion is considered to be most important [9-12] .It is believed that UPEC bacteria establish urinary tract infection and the presence of PapG on this organism could progress infection to a more severe form, called pyelonephritis [4, 9].Current treatment of acute urinary tract infection is limited due to the increasing rate of antibiotic resistance strains as well as adverse effects of vaccines [6, 13]. While several clinical trials of vaccine candidates have currently been tested against urinary tract infection in human, no suitable results have been found so far. Therefore, the design of new strategies in vaccine development can lead to an increase in vaccine efficacy [14, 15]. A variety of studies showed that the adsorption of vaccine candidate antigens on the surface of some bacterial cells, as a delivery system based on a live bacterial vector, can strongly stimulate immune responses [16, 17]. In the present study, the papG-AcmA fugen protein in *E. coli* was expressed and confirmed by western blotting. So, it could be considered as a candidate for vaccine production

MATERIALS AND METHODS

Construction of a recombinant PapG-AcmA expression vector

To bind the PapG protein to the surface of *Lactobacillus Reuteri*, the AcmA, as an

anchor protein, was linked to the PapG protein. The PapG-AcmA fusion gene segment containing histidine tag in the pEXA vector (at NdeI/EcoRI sites) was purchased from Bioneer Company (Bioneer, Korea). The pEXA/PapG-AcmA vector was digested with NdeI/EcoRI enzymes and after purification of the PapG-AcmA gene segment on the gel, the segment was ligated into the same digested pET21a vector. The fidelity of the pET21a/PapG-AcmA vector was confirmed with NdeI/BamHI enzymatic digestion and sequencing (Macrogene Institute, South Korea).

The expression of the recombinant PapG and PapG-AcmA proteins

The special *E. coli* strain Origami was used to express the recombinant PapG and PapG-AcmA proteins. The *E.coli* strain origami was cultured overnight in the LB medium. After refreshing the media once, the recombinant pET21a/PapG-AcmA and pET21a/PapG vectors were transformed into the *E. coli* strain Origami using a standard calcium chloride protocol. To express the recombinant PapG and PapG-AcmA proteins, the bacteria containing each plasmid were induced 4 hours using 1 mM IPTG induction. Furthermore, the protein expression was confirmed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The recombinant PapG and PapG-AcmA proteins were further confirmed with western blotting using anti-His antibodies.

Western blot analysis of the recombinant proteins

After IPTG-induction of the cells to express the recombinant proteins, SDS-page was performed to separate the proteins according to the molecular weights. Subsequently, proteins from the SDS-PAGE gel were electrophoretically transferred to the nitrocellulose membrane in Tris-glycin buffer containing 20% methanol. The unbound membrane sites were blocked by 3% TBS-BSA containing tween 20 (Tris-buffered saline-BSA 3% and tween 20). The membrane was then washed three times with TBS-T, and incubated with the 6 His-tag antibody HRP conjugate (Roche, Germany) for 2 hours. Afterwards, the membrane was washed again and incubated with of 3, 3-diaminobenzidine (DAB) substrate solution containing 1% H_2O_2. When a deep brown band appeared, the membrane was washed with distilled water to stop the enzymatic reaction.

Mass production and purification of PapG-AcmA proteins

Following the simultaneous expression of

PapG and PapG-AcmA proteins, the Ni-NTA column (Qiagen, USA) was used for mass purification. The *E.coli* cells expressing the recombinant proteins were centrifuged and the cell pellet was suspended in buffer A (20mM Tris-Hcl, 0.5M NaCl, 0.3% Triton X100, 1mM PMSF, 10mM imidazole, pH 8.0), and sonicated in 10 cycles of 40 seconds at 4°C by a sonicator (MSE, UK). The suspension was centrifuged (12000rpm, 20min, 4°C), the sample was loaded on a Ni-NTA column and purification was performed according to the manufacturer's standard protocol. Protein refolding was carried out by urea. After dialysis of purified samples, the concentration of each protein was detected via the Bradford method.

Endotoxin analysis of the products

The endotoxin level of purified PapG and PapG-AcmA was detected by the limulous amoebocyte lysate test (LAL test) according to the manufacturer's standard protocol (Thermoscientific, USA).

RESULTS

Construction of PapG and PapG-AcmA expression vectors

After construction of pET21a/PapG-AcmA and pET21a/PapG vectors, double digestion of recombinant plasmids was performed using NdeI/BamHI and NdeI/EcoRI restriction enzymes for the pET21a/PapG-AcmA and pET21a/PapG vectors, respectively. The results of agarose gel electrophoresis of digested plasmids showed the bands 945 bp and 629 bp for the PapG-AcmA and PapG gene segments, respectively (Figure 1). In addition, the results of sequencing confirmed both gene segments in the pET21a vector (data not shown).

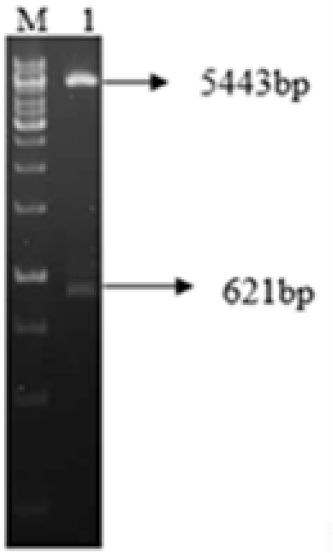

Figure 1. Enzymatic digestion of recombinant plasmids containing PapG and PapG.AcmA gene segments. Double digestion of pET21a/PapG vector with NdeI/BamHI enzymes showed a 629 bp band in electrophoresis. Lane M: DNA marker, Lane 1: PapG gene segment.

Prokaryotic expression of the recombinant PapG and PapG-AcmA proteins

The *E.coli* strain origami was used to express the recombinant PapG and PapG-AcmA proteins. After transformation and expression under 1 mM IPTG induction, SDS-page analysis indicated over expression of PapG (about 24 kDa)

and PapG-AcmA (about 34 kDa) proteins (Figure 2). Additionally, western blotting analysis with the anti His-tag antibody showed the bands of about 24 kDa and 34 kDa for PapG and PapG-AcmA proteins, respectively (Figure 3).

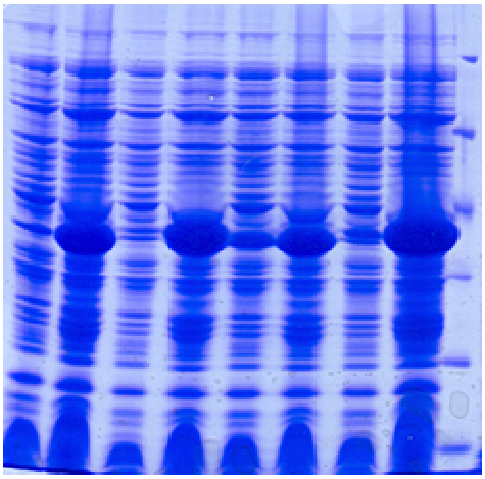

Figure 2. Expression of recombinant PapG.AcmA proteins under induction with 1mM of IPTG in E. coli origami strain. SDS-page analysis shows a 34 kDa recombinant PapG.AcmA fusion protein in lanes 2,4,6 and 8 versus un-induced samples at lanes 1,3,5 and 7. M: protein size marker.

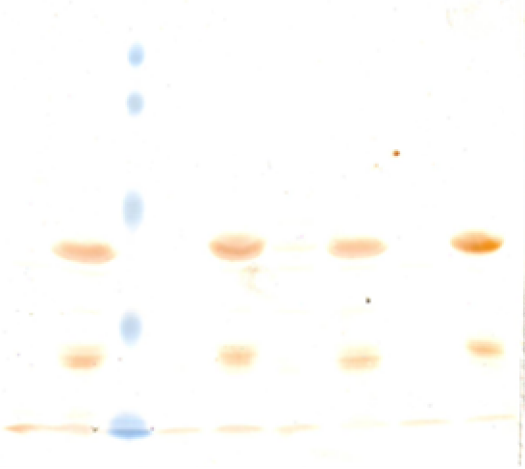

Figure 3. Western blot analysis of recombinant proteins. Analysis of PapG.AcmA protein versus anti-6 his antibody showed a 34 kDa band in western blotting. Lane 1 and 2: induced samples. Lane 3 un-induced sample as negative control. M: protein size marker.

Purification of the recombinant PapG and PapG-AcmA proteins

The Ni-NTA column was used to purify recombinant proteins. The purification results showed that after purification of the PapG and PapG-AcmA proteins and SDS-page analysis, a 24 kDa band for PapG and a 34 kDa band for PapG-AcmA were observed with minimal contamination with undesired proteins (Figure 4).

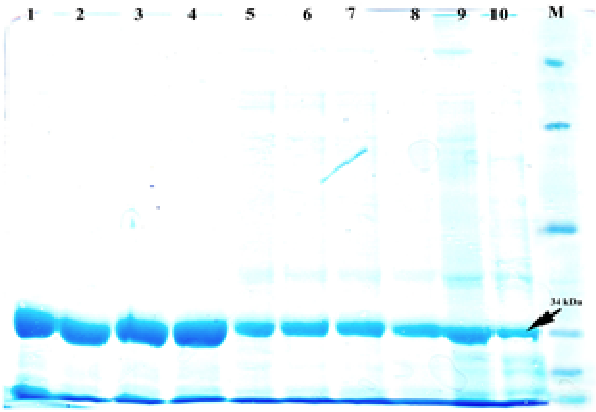

Figure 4. PapG.AcmA proteins via Ni-NTA column. Purification of recombinant PapG.AcmA fusion protein showed a 34 kDa band in SDS-page. Lane 1-10 show different fractions of purified PapG.AcmA fusion protein after purification via Ni-NTA column and analysis on SDS-page. M: protein size marker.

Endotoxin levels in the purified proteins

The results of endotoxin levels in the purified recombinant PapG and PapG-AcmA proteins using LAL test showed that the endotoxin level was less than 0.07 EU/ml.

DISCUSSION

E. coli remains one of the most common bacteria isolated from various human infections such as urinary tract infection, meningitis, and sepsis [1-3]. *E. coli* type P is one of the most important agents of urinary tract infection worldwide [9-12]. According to the various reports, the occurrence probability of urinary tract infection in women, children, the elderly and the immunocompromised patients is more than men [3, 10, 12]. The binding process in uropathogenic *E. coli* is facilitated through type 1 and P fimbriae [2, 9]. Unlike type 1 fimbria that mainly causes the infections of the lower urinary tract system, P fimbria leads to the ascending infections in the urinary tract and the basis for severe kidney infection pyelonephritis [2, 4, 9]. Nowadays, numerous studies have focused on urinary tract infection and a lot of researches have been performed in this area. Antibiotics are one of the well-known methods to reduce and prevent the acute urinary infection; developing an effective vaccine against the specific infection is considered as another possible strategy [6, 13, 15]. Since the binding step is very important in the colonization and infection process, inhibition of bacterial binding would be an effective strategy; a wide variety of vaccines focused on inhibition of bacterial binding through distinct molecules as targets [13-18]. In the present study, the recombinant protein PapG, as a critical adhesion molecule in the pathogenesis process of bacteria, was expressed. A variety of studies showed that PapG plays a

critical role in the attachment and appearance of the infection and inhibition of the molecule significantly inhibit the pyelonephritis infection [9-12].Therefore, we hypothesized that the use of *L. reuteri*, as a vaccine, modulates immune responses and increases vaccine potency. In fact, through stimulation of the innate immune system and AcmA receptors [17-19], L.reuteri provides a milieu in which lymphocytes are able to recognize PapG in the inflammatory condition and more immunogenic form. In this light, the recombinant protein PapG was first produced and then confirmed in SDS-page and western blotting. PapG, as an immunogenic form, was displayed on the surface of L.reuteri. For this purpose, the AcmA protein, as an anchor molecule, was used. A lot of studies showed that AcmA can link to a protein on the surface of *Lactobacillus* sp [17, 18]. In this regard, AcmA protein was used as a linker molecule in this study. To link the molecule to the surface of *L.reuteri*, the PapG protein was fused at the gene level to produce PapG-AcmA as a fusion protein for linkage of PapG to the surface of *L.reuteri*. Several studies showed that AcmA acts as a suitable linker for ligation of various proteins on the surface *of Lactobacillus* sp [19, 20]. Various studies have so far been conducted on the presentation of antigens at the surface of bacteria such as *lactobacillus lactis*. In an experience, Helicobacter pylori urease subunit B gene E fragment with anchor sequence of *Staphylococcus aureus* (spax) was cloned into a PAMJ399 expressing vector, expressed under a p170 promoter *in lactobacillus lactis*, and confirmed by western blot analysis. These results were the first report of a surface presentation system in lactic acid bacteria to provide an oral vaccine against *Helicobacter pylori* [21].

Jian-kui Liu et al. in 2009 presented *E. coli* PgsA and F41 antigens on the surface of *lactobacillus casei* and antigen display was confirmed by immunoblotting, immunoflurosense and flowcytometry. In addition, oral administration of surface displayed *lactobacillus* to mice caused an increase in the secretory IgA titer and protection in the experimental challenge [15]. In the present study, we could produce *L. reuteri* bacteria displaying the PapG protein as a vaccine candidate for further study.

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