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Comparison of the immunogenicity between bacterial ghost and formalin-killed bacteria for *Vibrio vulnificus*

Se Ryun Kwon†

Department of Aquatic Life Medical Sciences, Sunmoon University, Asan-si, Chungnam 336-708, Korea

Vibrio vulnificus ghosts (VVG) were generated using a mobilizable vector including a thermosensitive expression cassette by conjugation. The vaccine potential of VVG was investigated in mouse. Mice immunized with VVG showed significantly higher antibody titer than those with formalin-killed *V. vulnificus*. The present study supports the conceptive usefulness of bacterial ghosts as vaccine candidates.

Key words : Bacterial ghost, *Vibrio vulnificus*, Immunogenicity, ELISA

Vibrio vulnificus is a gram-negative, rod-shape bacterium that is commonly found in warm waters with salinity between 1.6% and 2.3% (Kelly 1982; Tamplin *et al*., 1982). It causes severe wound infections, gastroenteritis, and septicemia in eels (Tison *et al*., 1982) and humans (Park *et al*., 1991; Chuang *et al*., 1992; Hlady *et al*., 1993). In addition, mice experimentally infected by subcutaneous injection with *V. vulnificus* exhibit extensive edema and tissue necrosis at the initial site of infection and a rapidly developing, fatal septicemia (Bowdre *et al*., 1981) similar to that observed during human disease.

The outer membrane proteins of pathogenic bacteria have been studied in relation to inducing protective humoral and cell-mediated immunities (Heckels *et al*., 1989; Sengupta *et al*., 1992; Lutwyche *et al*., 1995; Kawai *et al*., 2004). Recently, Jung *et al*. (2005) reported that vaccination with major outer membrane protein of *V. vulnificus* resulted in protective antibodies in the mouse infection experiment. In contrast to capsular polysaccharides or LPS, outer membrane proteins are T-cell-dependent antigens and can elicit strong and long-lasting humoral immune responses (Hansen *et al*., 2008). To produce effective and protective antibodies using outer membrane proteins, the conformation of the proteins in vaccine formulations, irrespective to subunit vaccine or whole cell vaccine, should not be modified by chemical or physical forces, as B cell recognize native form of antigen epitopes. However, traditional inactivation of bacteria by heat or formalin may influence the physico-chemical characteristics of surface antigens, and immune responses against the modified antigens may not be protective against live bacteria (Kwon *et al*., 2006 & 2007; Lee *et al*., 2008; Kwon *et al*., 2009; Tu *et al*., 2009).

Bacterial ghosts could be produced by the expression of bacteriophage PhiX174 lysis gene E which leads to the formation of small transmembrane channels.

†Corresponding author : Se Ryun Kwon
Tel : +82-41-530-2289, Fax : +82-41-530-2917
E-mail : srkwon@sunmoon.ac.kr

Through the pore, cytoplasmic materials were expelled out of the cell. Bacterial ghosts have functional and antigenic envelope structures of their living counterparts in a naive state since this genetic inactivation process is not known to cause any physical or chemical denaturation of the bacterial surface structures. In the present study, we have generated *V. vulnificus* ghosts (VVG) using a conjugation method, and compared the immunogenicity of the VVG with formalin-killed cells in mouse.

Materials and methods

Bacterial strain and culture condition

Vibrio vulnificus provided by National Fisheries Research & Development Institute, Korea, was grown in tryptic soy broth (TSB, Difco) containing 1.5% NaCl at 27℃. *Escherichia coli* SM10λ*pir* was used as a donor and was cultured in Luria Broth (LB, Difco) containing 30 ㎍/㎖ kanamycin (Sigma). Transformed *V. vulnificus* was selected in thiosulphate-citrate-bile-sucrose agar (TCBS, Difco) containing 15 ㎍/㎖ tetracycline (Sigma) and grown in LB containing 15 ㎍/㎖ tetracycline.

Construction of mobilizable ghost plasmid

Ghost cassette containing *BamH* Ⅰ and *Pst* Ⅰ enzyme site was obtained by PCR from pλP_R-c Ⅰ-Elysis constructed by Kwon *et al.* (2006) using the primers LC Ⅰ Pst-F (5'-CTGCAGGACCAGAACACCTTGCCGAT-3') and EBamH-R (5'-GGATCCACATTACATCACTCCTTCCG-3'). PCR amplifications were performed for 1 cycle of 3 min at 95℃, 30 cycles of 30 s at 95℃, 30 s at 55℃, 1 min at 72℃, with a final extension step of 7 min at 72℃. PCR reactions were performed using the iCycler thermal cycler (Bio-Rad). Each amplified PCR product was visualized on 0.7% agarose gels stained with ethidium bromide, purified with gel extraction kit (Nucleogen) and cloned into pGEM-T easy vector (Promega). After *BamH* Ⅰ and *Pst* Ⅰ digestion of, ghost cassette was ligated into the digested pRK415 plasmid, a mobilizable vector. The constructed vector was named as pRK-λP_R-c Ⅰ-Elysis (Fig. 1).

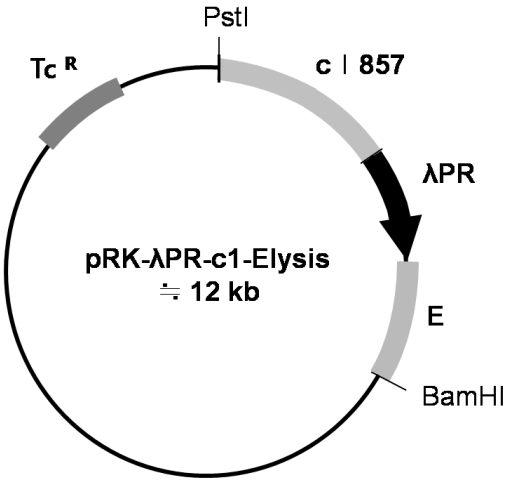

Fig. 1. Partial restriction map of pRK-λPR-cI-Elysis. The plasmid used for the production of *V. vulnificus* ghosts harbors the E lysis cassette, consisting of the lysis gene E (E), the leftward Lamda promoter (λPR), the temperature sensitive repressor cI857 (c Ⅰ857), and tetracycline resistance gene (TetR).

Conjugation of *V. vulnificus*

E. coli SM10λ*pir* harboring pRK-λP_R-c Ⅰ-Elysis was used as a donor for transformation of *V. vulnificus*. Donor cells were suspended to a density of 10^8 CFU/㎖, and *V. vulnificus* cells were suspended to a density of 10^7 CFU/ml Equal volumes (100 ㎕) of donor and recipient cells were mixed and placed onto LB agar plate. After

16 h of incubation, the mating product was resuspended in 1 ml of LB medium and transconjugants were selected by plating on TCBS agar containing 15 ㎍/ml tetracycline. Transconjugants were verified by plasmid preparation.

Preparation of *V. vulnificus* ghosts (VVG)

V. vulnificus transformant was cultured at 27℃ before an optical density at 600 nm of bacterial culture reach 0.3. At the point, the temperature was elevated to 42℃ for the induction of lysis gene expression. Growth and lysis of bacterial cultures were monitored. At the end of lysis, ghosts were harvested, washed and resuspended in PBS and then lyophilized. At the end of lysis, the efficacy of bacterial killing was estimated by plating 10 ㎕ of 100 times dilutions of lyophilized VVG on LB agar containing 15 ㎍/ml tetracycline and a 100% killing efficiency was confirmed as no colony-forming unit.

Preparation of formalin-killed *V. vulnificus* (FKC)

V. vulnificus was grown for 24 hr at 27℃ in TSB containing 1.5% NaCl and added formalin at the final concentration of 0.5% for FKC preparation. After 24h incubation, cells were washed three times with PBS and resuspened in 10 ml of PBS. The suspensions were streaked on tryptic soy agar containing 1.5% NaCl for checking sterility and stored at 4℃ until use.

Immunization

Eight-week-old ICR mice were purchased from Daehan Biolink co., LTD. The experimental groups were divided three groups. The mice were injected i.p. with 200 ㎍ of *V. vulnificus* ghosts (VVG group), formalin-killed *V. vulnificus* (FKC group),or PBS alone (Control group). The animals were boosted at 2 weeks after the first immunization with the same dose used in the first injection. Two weeks after the boosting, 2 mice in each group were sacrificed and blood specimens were collected from the heart for the further assay.

Enzyme linked immunosorbant assay (ELISA)

The flat-bottomed ELISA plates were coated with 50㎕ of *V. vulnificus* FKC (4 ㎎/ml) in PBS for 2 hr at 60℃. The plates were then washed thoroughly with PBST (PBS containing 0.1% Tween 20) and blocked with 200 ㎕ of 2% BSA in PBS for 1 hr at 37℃. Subsequently, the plates were washed thoroughly with PBST and incubated with 75 ㎕ of mouse antiserum from each group at 37℃ for 30 min. The test sera were serially diluted two-fold at 1:50 of initial dilution ratio. The plates were washed with PBST and incubated with 75 ㎕ of goat anti-mouse IgG conjugated with alkaline phosphatase (1:1000, Santa Cruz) for 30 min at room temperature. The plates were washed with PBST three times and developed with the substrate p-nitrophenyl phosphate in substrate buffer at dark room. After 30 min incubation, the optical density was measured at 415 nm using an automated ELISA reader (Bio-Rad). ELISA data was analyzed by the Student's *t*-test and the chi-square test with a significance level of $P = 0.05$.

Results and discussion

Transformation of *V. vulnificus* was accomplished successfully by conjugation with the mobilizable vector including a ghost cassette. Hamashima *et al*., (1995)

reported that *Vibrio parahemolyticus, Vibrio alginolyticus*, and *Vibrio cholerae* can be easily transformed by electroporation. However, *V. vulnificus* couldn't accept a plasmid by a chemically transformation or electroporation despite of repeated trails. By that reason, conjugation vector based-bacterial ghost plasmid, pRK-λP_R-c Ⅰ-Elysis was constructed (Fig. 1). Generation of ghosts was performed by increasing the incubation temperature up to 42℃. The lysis was observed within 2 hr after temperature elevation and completed 6 hr after induction of *E* gene expression (Fig. 2). In aquatic bacterial ghosts under control of the temperature sensitive promoter repressor system, the production of *Edwardsiella tarda* ghost and *V. anguillarum* ghost was accomplished within 16 hr and 10 hr, respectively (Kwon *et al*., 2005 & 2009). Thus, the ghost generation kinetics of *Vibrio* species was higher than that of *E. tarda*.

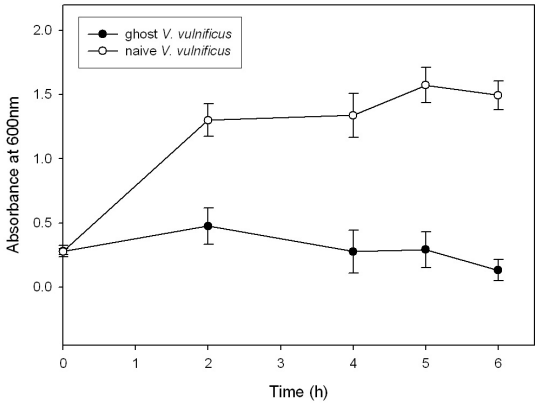

Fig. 2. Growth and lysis curves of *V. vulnificus* harboring plasmid pRK-λP_R-cI-Elysis by temperature induction of gene *E* expression. At time zero, the cultures were shifted from 27℃ to 42℃.

To address the immunogenicity of *V. vulnificus* ghosts (VVG) generated, the antibody titer for the mouse immunized with VVG was compared with that with formalin-killed *V. vulnificus* (FKC). In the present study, mice immunized with VVG showed significantly higher antibody titer than mice immunized with FKC (Fig. 3). Similar results were reported in tilapia and olive flounder immunized with *E. tarda* ghost (Kwon *et al*., 2006 & 2007),suggesting that VVG has more optimal antigenicity for inducing protective immune responses than FKC. In mammals, pigs immunized with ghosts made from *Actinobacillus pleuropneumoniae* (App) or with formalin- inactivated App were found to be protected against clinical disease in both vaccination groups whereas colonization of the lungs with App was only prevented in ghost-vaccinated pigs (Huter *et al*., 2000).

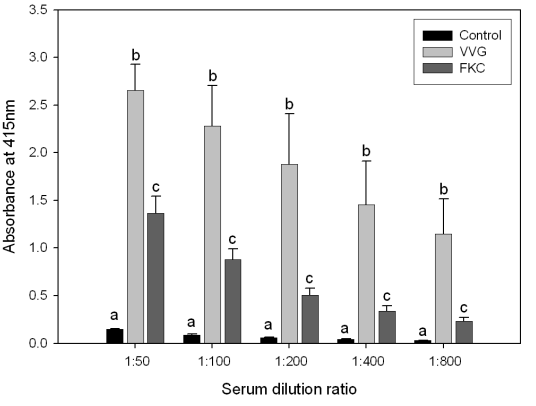

Fig. 3. ELISA antibody titer in the serum from the male mice immunized with *V. vulnificus* ghosts (VVG), formalin-killed *V. vulnificus* (FKC) or PBS alone (Control). Control serum was derived from the mice immunized with PBS alone. Bars with same letters within each dilution ratio did not differ significantly at $P<0.05$.

It has been suggested that protective epitopes are expressed in pathogenic bacteria, which elicit protection regardless of the somatic serotype of the infecting strains

(Kasten *et al*., 1995). However, in inactivated vaccine preparations, these epitopes might be destroyed by chemical and physical stresses caused by conventional inactivation procedures (Melamed *et al*., 1991; Nencioni *et al*., 1991; Ferguson *et al*., 1993). Although live vaccines seem to be favorable by presenting all antigen epitopes of a cell in their native conformation, it is also widely agreed that current live vaccines may also have potential risks associated with unwanted revert to virulence. However, bacterial ghosts not only experience little physical or chemical denaturation but also don't recover their virulence.

The present results suggest that the effective delivery of a foreign plasmid is possible for *V. vulnificus* based on the conjugation method and also that utilization of the bacterial ghosts for this bacterial species could be a potential means to develop a non-living animal vaccine of which efficacy is superior for that of the FKC version. From this basis, further studies are needed in order to probate the protective effect in more detailed fashion.

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