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Full Length Research Paper

Surface display of multi-epitopes synthetic gene of *Mycobacterium tuberculosis* on *Salmonella typhi* Ty21a using a pgsA anchor protein

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We have developed a recombinant *Salmonella typhi* Ty21a vaccine candidate (rSTvacIII) displaying a multi-epitopes mycobacterial antigen called the VacIII (containing ubiquitin and 4 immunogenic mycobacterial epitopes of ESAT-6, PhoS1, Hsp 16.3 and MTB 8.4 genes) on the surface of *S. typhi* Ty21a (Ty21a) using PgsA protein as a carrier. The expression of VacIII protein on the surface of Ty21a was verified by western blot. Immunization in BALB/c mice with rSTvacIII induced strong Th1-type responses based on high level of *in vitro* IFN-γ but low IL-4 secretion. Since the protective immunity against tuberculosis is mediated by Th1-type response, the rSTvacIII have potential as effective vaccine candidate against tuberculosis.

Key words: VacIII antigen, PgsA anchoring motif, tuberculosis, *Salmonella typhi* Ty21a.

INTRODUCTION

Displaying foreign proteins on bacterial surface has gained increasing attention in many biotechnological and medical applications (Samuelson et al., 2002; Lee et al., 2003; Rutherford and Mourez, 2006). This technology has been used for enzyme libraries screening (Kim et al., 2001; Becker et al 2004), the development of bioadsorbents (Xu and Lee, 1999) and the construction of surface display bacterial vaccines (Haddad et al., 1995; Lee et al., 2000; Grode et al., 2002; Kramer et al., 2003). A number of studies have shown that, antigens displayed on the surface of bacterial vaccine able to induce higher immunological responses compared with antigens secreted intracellularly (Lee et al., 2000; Grode et al., 2002; Stover et al., 1993).

Foreign proteins are displayed on the bacterial surface by fusing them with a carrier protein. Carrier protein is a protein normally associated with a cell membrane of an organism (Lee et al., 2003). A number of carrier proteins such as ice nucleation protein (Inp) (Kim et al., 2001; Lee et al., 2000; Kwak et al., 1999), outer membrane protein (Omp) (Haddad et al., 1995) and autotransporter (Rutherford and Mourez, 2006; Kramer et al., 2003) have been used to display foreign protein on bacterial cell surfaces. The use of different carrier proteins frequently cause different physiological effects on host cells including growth defects and destabilization of the outer membrane (Lee et al., 2003; Narita et al., 2006).

The efforts to develop better carrier proteins are still in progress and new carrier proteins have been developed recently including PrsA (Kim et al., 2005) and PgsA (Ashiuchi et al., 2004; Narita et al., 2006). PgsA protein is a carrier protein derived from poly-γ-glutamate (PGA) synthetase complex of *Bacillus subtillis* (Ashiuchi et al., 2004). Narita et al. have shown that, PgsA protein is able to display proteins as large as 77 kDa in active form on the surface of *Escherichia coli* (Narita et al., 2006).

Salmonella typhi Ty21a (*S. typhi* Ty21a) is a live attenuated vaccine for typhoid fever (Curtiss, 2002). This vaccine strain has many advantages such as proven

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Table 1. List of primers and synthetic peptides.

Name of primer	Sequence of nucleotide
Mutant *Bam*HI reverse	5' GCC GAT GAG GAC CCT GTT GAT GCC G 3'
Mutant *Bam*HI forward	5' CGG CAT CAA CAG GGT CCT CAT CGG C 3'
VacIII reverse	5' TCG TAA AGC TTA TGC AGA TCT TCG TT 3'
VacIII forward	5' TCGTGGTACCCAGATCTTCGTTAAGACCC 3'
Mtb 8.4a	DPVDAVINTTCNYGQVVAA
Mtb 8.4b	GQVVAALNATDPGAAAQFN
Mtb 8.4c	AAAQFNASPVAQSYLRNFLA
Mtb 8.4d	NFLAAPPPQRAAMAAQLQAV
Mtb 8.4e	ALQAVPGAAQYIGLVESVAGSCNNY

safety, inexpensive to produce and the ability to express protective antigens of multiple pathogens (Kochi et al., 2003). Moreover, Lundin et al. (2002) has found that, *S. typhi* Ty21a induces robust Th1 responses, characterized by a high production of IFN-γ and no IL-10 or TGF-β. The Th1 responses are also crucial in protective immunity against TB infection. Accordingly, this vaccine strain has the potential to be developed further as an effective vaccine against TB.

In the present study, the PgsA protein is used as carrier protein to display multi-epitopes of MTB proteins (VacIII protein) on the surface of *S. typhi* Ty21a. The N-terminal of the VacIII protein was fused to the C-terminal of PgsA gene under the control of the HCE promoter (Poo et al., 2002).

The VacIII is a multi-epitope synthetic gene, constructed by assembly PCR, which consists of UbGR (Ubiquitin) and seven T-cell epitopes of MTB proteins: ESAT-6 (P1), ESAT-6 (P2), Phos1 (65-83), Phos1 (129-137), Phos1 (166-175), Hsp 16.3 and Mtb 8.4) (Fang et al., 2006).

MATERIALS AND METHODS

Bacterial strain and plasmids

E. coli JM109 *recA1 supA sbcB15 hsdR4 rpsL thi* Δ*(lac-proAB) F'[traD36 proAB*$^{+}$*lacIq lacZ*Δ*M15, Escherichia coli* TOP10 F^{-} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G (Invitrogen, CA, USA) and *S. typhi* Ty21a (BERNA, Swiss Serum and Vaccine Institute Berne Switzerland). Plasmid pT(II)(*Nde*I)-pgsA-HBV was obtained from Prof. Kim Chul-Joong (National Research Laboratory, College of Verterinary Medicine, Chungnam National University, 220 Gungdong, Yusung, Daejeon, Korea). This plasmid contains the HCE promoter and pgsA surface display system. VacIII gene was isolated from pTInak-VacIII (Fang et al., 2006).

Primers, synthetic peptides and recombinant protein

Primers were synthesized by First BASE Laboratories Pte. Ltd. Synthetic MTB 8.4 peptides (Table 1) were synthesized by Genemed Synthesis, Inc. (CA, USA). Recombinant VacIII protein was kindly given by Dr. Fang Chee Mun (Fang et al., 2006).

Mice

BALB/c (H-2d) female mice age 6 to 12 weeks-old was obtained from the Animal House, USM Health Campus. The mice were kept in sterile condition and given sterile water and standard pellet food

Construction of surface display S. typhi Ty21a expressing VacIII protein

The construction of surface display plasmid pT(II)(NdeI)-pgsA-VacIII is summarized schematically in Figure 1. A VacIII surface display expression plasmid (pT(II)(*Nde*I)-pgsA-VacIII) was prepared by first digesting plasmid pTInakVacIII, using restriction enzymes (RE) *Kpn*I and *Hin*dIII to obtain VacIII DNA fragment (Figure 2). The VacIII DNA fragment (771 bp in size) was separated by 0.8% agarose gel electrophoresis and gel purified using QIAquick gel extraction kit (Qiagen, USA).

Then, the purified *Kpn*I-*Hin*dIII VacIII DNA fragment was ligated using T4 DNA ligase (Roche, Germany) with *Kpn*I-*Hin*dIII digested pCR®2.1-TOPO® to generate pTOPO-VacIII. The ligation mixture was then transformed into TOP10 *E. coli* competent cells by standard $CaCl_2$ method (Sambrook et al., 1989). For the cloning of VacIII into pT(II)(*Nde*I)-pgsA, plasmid pTOPO-VacIII was digested with *Bam*HI-*Hin*dIII followed by gel purification. The gel purified VacIII DNA fragment was ligated into *Bam*HI-*Hin*dIII digested pT(II)(*Nde*I)-pgsA to generate pT(II)(*Nde*I)-pgsA-VacIII and transformed into JM109 *E. coli*. The success of the ligation process was confirmed using DNA sequencing using VacIII forward and VacIII reverse primers (Table 1). After plasmid extraction, pT(II)(*Nde*I)-pgsA-VacIII was transformed into competent *S. typhi* Ty21a by $CaCl_2$ method. The recombinant *S. typhi* Ty21a containing pT(II)(*Nde*I)-pgsA-VacIII was designated as recombinant STvacIII (rSTvacIII).

Expression study of rSTvacIII

To determine the performance of the HCE promoter in pgsA-VacIII protein expression, rSTvacIII was cultured for 16, 18 and 23 h was prepared according to previous method (Poo et al., 2002). In order to confirm that, the PgsA-VacIII protein was displayed on the surface of the bacteria, acidic glycine extraction was done according to a previously described method (Logan and Trust, 1983). Briefly, a single colony was inoculated in 200 ml tryptic soy broth containing 100 µg/ml ampicillin and incubated in incubator

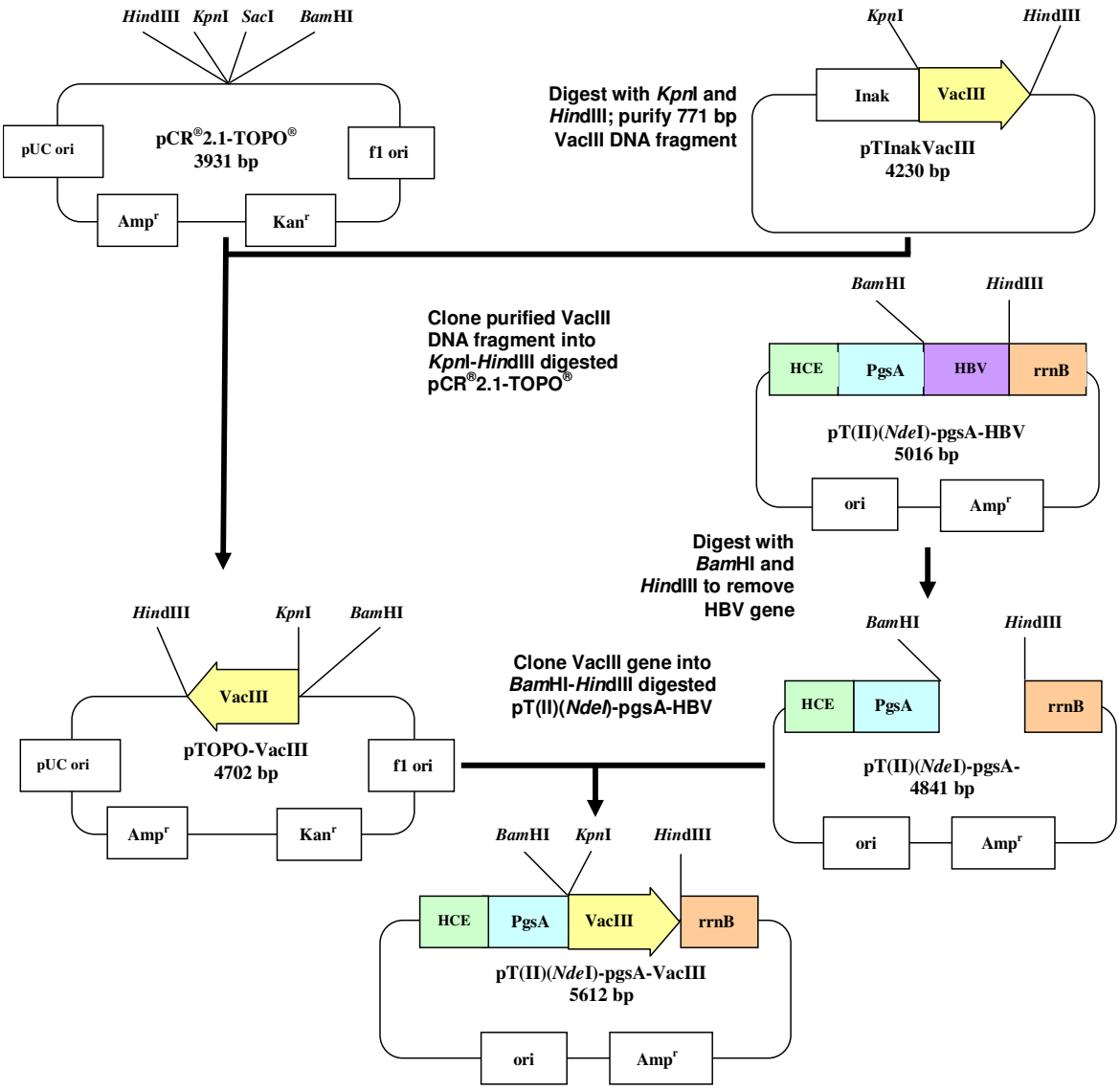

Figure 1. The construction of surface display plasmid pT(II)(*Nde*I)-pgsA-VacIII which has been used throughout this study. Digestion of pTInakVacIII with *Kpn*I and *Hin*dIII restriction enzymes produced two fragments 771 and 3459 bp. The 771 bp (VacIII gene) was ligated with *Kpn*I-*Hin*dIII digested pCR®2.1-TOPO® to produce pTOPO-VacIII. Then the pTOPO-VacIII was subjected to *Bam*HI and *Hin*dIII digestion resulted in two fragments of 771 bp (VacIII) and 3.9 kb (pCR®2.1-TOPO® vector). The gel purified VacIII DNA fragment was ligated into *Bam*HI-*Hin*dIII digested pT(II)*(Nde*I)-pgsA-HBV to generate the surface display plasmid pT(II)(*Nde*I)-pgsA-VacIII. The plasmid was transformed into *S. typhi* Ty21a and named as rSTvacIII.

shaker (New Brunswick Scientific, USA) at 37°C, 200 rpm, overnight. The overnight culture was poured into a 50 ml Falcon tube and harvested by centrifugation at 3000×*g*.

Then the cell pellets were resuspended in 500 µl of 0.2 M glycine hydrochloride, pH 2.2, briefly vortexed and incubated at room temperature for 15 min. The intact cells were removed by centrifugation at 12,000×*g* for 15 min at 8°C and the supernatant was transferred into a sterile tube. The pH of the supernatant was adjusted with 3 M NaOH to pH 7.4.

Two volumes of ice-cold absolute ethanol were added to the supernatant prior to incubation at -20°C overnight. After incubation, the mixture was centrifuged at 12,000×*g* at 8°C for 30 min. The supernatant was discarded and the pellets were resuspended in 30 µl of 10 mM Tris buffer pH 7.4 and 1 mM PMSF was added into the suspension.

SDS-PAGE and western blot

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli (1970). Protein samples (5 to 50 µg) were mixed with sample buffer with ratio 1:1 v:v in microfuge tubes. The protein samples were denatured by incubating for 5 min in boiling water. The samples were allowed to cool prior to loading into wells of the SDS polyacrylamide gel. Prestained protein ladder was used as

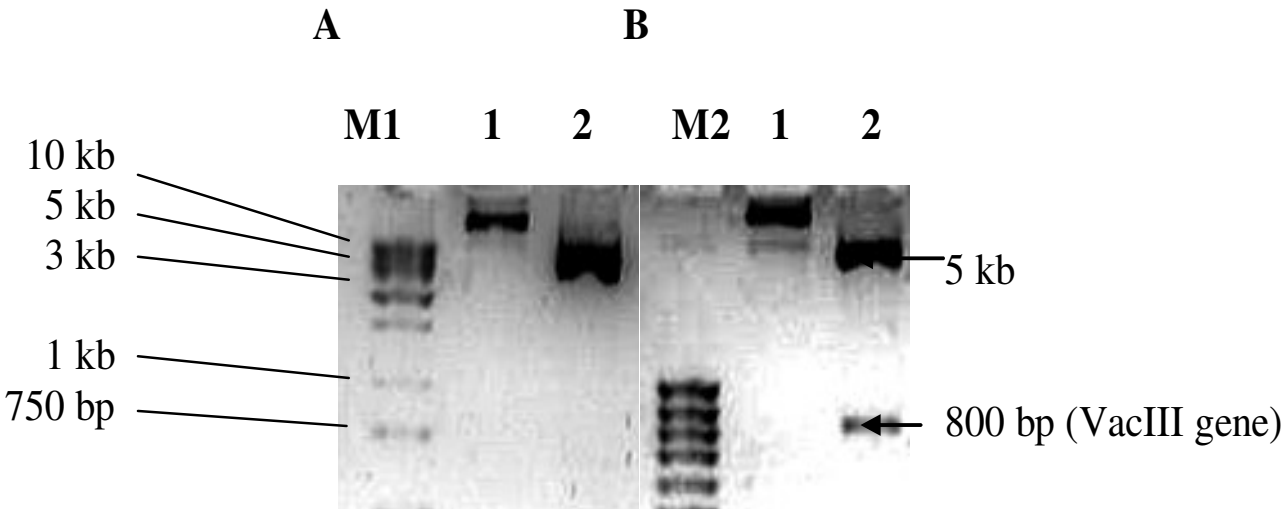

Figure 2. The 1.6% agarose gel electrophoresis showed restriction enzyme (RE) analysis of pT(II)(*Nde*I)-pgsA-HBV and pT(II)(*Nde*I)-pgsA-VacIII using RE *Bam*HI and *Hin*dIII. A) pT(II)(*Nde*I)-pgsA-HBV uncut (lane 1) and *Bam*HI-*Hin*dIII digested (lane 2) B) pT(II)(*Nde*I)-pgsA-VacIII uncut (lane 1) and *Bam*HI-*Hin*dIII digested (lane 2). M1: 1 kb DNA Ladder (Fermentas, Lithuania), M2: 100 bp DNA Ladder (Fermentas, Lithuania). The gel was stained using Ethidium Bromide.

protein molecular weight marker.

Protein from SDS-PAGE was transferred onto nitrocellulose membrane using a trans-blot semi-dry transfer cell according to manufacturer's instructions (BIORAD, USA). After running SDS-PAGE, the resolving gel was taken with caution and soaked into transfer buffer. At the same time extra thick whatman paper and nitrocellulose membrane were soaked in transfer buffer. Then the extra thick whatman paper was placed on a blotting chamber with the polyacrylamide gel on the top. Nitrocellulose membrane was placed on the polyacrylamide gel followed by extra thick paper. A 10 ml glass pipette was rolled on the extra thick paper to remove air bubbles. Trans-Blot semi-dry transfer cell was closed by placing the upper lid on the second extra thick paper. The transfer process was carried out at 13 volts for 25 min and the blotted nitrocellulose membrane was used for immunoassay.

For immunoassay, the nitrocellulose membrane was washed with 1X PBS for 10 min and horizontally rotated on a belly dancer. The membrane was incubated in 3% skimmed milk for 1 h followed by washing with PBS-T20, 3 times for 10 min. Then, the nitrocellulose membrane was incubated with primary monoclonal antibody HYB 76-8 (generously donated by Ida Rosenkrands, Statens Seruminstitut, Copenhagen, Denmark) (this antibody was diluted 1:250 in 1X PBS) and incubated at 37°C for 2 h. HYB 76-8 binds to the amino acid sequence TEQQWDFAGI located in the ESAT6 (P1) epitope.

Preparation of rSTvacIII for vaccination

The bacterial strain was prepared for oral vaccination according to method described in previous report (Lee et al., 2000). Bacteria was grown for overnight in tryptic soy broth without dextrose containing 100 µg/ml ampicillin at 37°C until the OD 600 nm was 1.0, which is approximately equal to 2 X 10^9 CFU (estimated by plating dilutions). The cells were harvested by centrifugation at 3000×*g* for 10 min and washed three times with 1X PBS (pH 7). The cells suspension was diluted in PBS to obtain a concentration 2 X 10^9 cells/ml prior to immunization.

Immunization

Intragastric stainless steel gavage needle with a smooth ball tip attached to its distal end was used to immunize the mice. A 0.1 ml volume of 3% sodium bicarbonate was first administered to the mice 5 min before the immunization to neutralize stomach acidity. The needle was inserted through the mouth and then to the oesophagus at depth of approximately 2 cm. A 0.2 ml volume of 2X10^9 CFU of rSTvacIII or control (*S. typhi* Ty21a harbouring pT(II)(NdeI)-pgsA- and designated as rST) resuspended in PBS was inoculated into the mouse's oesophagus. Each group was containing 4 to 6 mice. Second immunization was given 2 weeks after the first immunization.

For antibody isotyping analysis, two weeks after boosting, the tail blood of vaccinated and control mice were taken and kept at 4°C overnight. The sera were harvested from the tail blood by centrifugation at 1500 × *g* at 8°C and then stored at -20°C until use.

For cytokine analysis, two weeks after the last administration of vaccine or control, vaccinated mice were sacrificed. Spleens of the mice were taken aseptically and used for splenocytes preparation.

Splenocytes preparation

In order to prepare a single cell suspension, the collected spleens were placed on a sterile mesh stainless steel wire screen and crushed using a sterile disposable plastic syringe piston. The cell suspension was resuspended in a sterile Petri dish containing 5 ml ice cold RPMI 1640 and transferred to a syringe containing a sterile cotton. 2 ml of media was used to rinse the Petri dish and transferred to the same syringe.

The filtered cell suspension was collected in a sterile 15 ml Falcon tube and then centrifuged at 300×g, 10°C for five minutes. The cells were washed twice with 10 ml of fresh sterile medium and resuspended in five ml ACK lysis buffer to lyse the red blood cells. The resuspended cells were incubated for 5 min on ice followed by the addition of 45 ml RPMI 1640 medium prior to centrifugation at 300×g, 10°C for 5 min. The cell pellet was washed twice by centrifugation-resuspension at 300×g, 10°C for 5 min using 2 ml of

RPMI and lastly, the cells were resuspended in 1 ml complete RPMI (containing 2 mM L-glutamine, 25 mM HEPES, 1% penicillin-streptomycin and 10% FBS). The number of viable cells was estimated using tryphan blue staining and adjusted to 2 x 10^6 cells/ml.

Quantification of IFN-γand IL-4 in the culture supernatant by ELISA

For measurement of IFN-γ and IL-4 cytokines production, 100 µl of splenocytes (2 x 10^6 cells/ml) were placed in each 96-well flat bottom NunC-Immunoplate. The cultures were stimulated with 100 µl (10 µg/ml) of MTB 8.4 pooled peptides or recombinant VacIII protein per plate. Concanavalin A (final concentration is 5 ug/ml) and medium alone were also used as a non-specific stimulator and negative control, respectively. The splenocytes culture was incubated at 37°C with 5% CO_2. After 48 h, 100 µl of culture supernatant from each well was carefully aspirated and kept in -70°C until use. For this assay, OptEIA™ mouse IFN-γ set and IL-4 set (Pharmingen, USA), respectively were used according to manufacturer recommendation. Before the assay was performed, the culture supernatant was diluted 1:3 with assay diluent (10% FBS in 1X PBS). To ensure reproducibility of results, the assay was done in triplicate.

Measurement of IgG1 and IgG2a in mice sera by ELISA

Sera obtained from vaccinated mice were used to measure the level of specific IgG1 and IgG2a. The test was done using 96 well plates which coated with 100 µl of rVacIII (at 2 µg/ml final concentration diluted in carbonate-bicarbonate buffer) per well. The plate was sealed and incubated in 4°C overnight. After removing the rVacIII protein solution, the wells were incubated with 200 µl of blocking buffer (10% FBS) for 1 h at 37°C. The buffer was removed and 100 µl of mice sera (diluted 1:100 in 1X PBS 0.05% Tween 20) was added into each well and the plate incubated for 2 h at 37°C. The sera was removed and 100 µl of horseradish-peroxidase conjugate goat anti mouse IgG1 or IgG2a (diluted 1:3000 in 1X PBS 0.05% Tween 20) was added per well and the plate incubated for 1 h. After removal of the conjugate solution and washing thrice with buffer, the reaction was developed by adding 100 µl of substrate solution (Tetramethylbenzene (TMB), BD Biosciences, San Jose, CA) per well and the plate was incubated 30 min in dark. The reaction was stopped by adding 50 µl of 2N H_2SO_4 into each well. The level of IgG1 and IgG2a in mice sera was determined by measuring the optical density (450 nm). The IgG2a:IgG1 ratio was calculated by dividing the optical density of IgG2a to IgG1.

Statistical analysis

Significant differences among groups were assessed using non-parametric Kruskal-Wallis test (SPSS 11.0.1).

RESULTS

Construction of rSTvacIII

The VacIII gene has successfully cloned into pT(II)(*Nde*I)-PgsA- to generate pT(II)(*Nde*I)-PgsA-VacIII. The pT(II)(*Nde*I)-PgsA-VacIII plasmid was screened by double digestion using *Bam*HI and *Hin*dIII and resulted in two fragments 800 bp (VacIII DNA fragment) and 5 kb (pT(II)(NdeI)-PgsA vector) (Figure 2).

Expression study of rSTvacIII

To determine the performance of HCE promoter in pgsA-VacIII protein expression, rSTvacIII was cultured for 16, 18 and 23 h according to method by Poo et al. (2002) with slightly modification. Pellet and supernatant from cell lysate of rSTvacIII were subject to SDS-PAGE and western blot. Bands similar in molecular weight (between 70 to 100 kDa) were detected in pellet from cell lysate of rSTvacIII cultured for 16, 18 and 23 h (Figure 3a). The band sizes ranging from 70 to 100 kDa are similar to the estimated size of PgsA-VacIII protein (75 kDa). No band was detected in the negative control (rST). This result showed that, the PgsA-VacIII protein had been successfully expressed by rSTvacIII. However, the expression of PgsA-VacIII protein in rSTvacIII was reduced after 16 h. Accordingly, the rSTvacIII surface protein extraction was carried out using the 16 h rSTvacIII culture.

Western blot analysis of rSTvacIII surface protein also showed the presence of one band of the expected size of PgsA-VacIII protein (Figure 3b). These results suggested that, rSTvacIII was able to express and display pgsA-VacIII protein on its surface.

Oral vaccination of mice vaccinated with rSTvacIII induced mixed Th1 and Th2 type responses with more to Th1-type response

The immunogenicity of rSTvacIII studied in mice was based on cytokine production *in vitro* and serum IgG antibody subclasses. Accordingly, two weeks after the last vaccination, spleens and sera from vaccinated mice were taken for analyses. Cytokine production was measured in culture supernatant of splenocytes stimulated with MTB 8.4 pooled peptides and recombinant VacIII protein (rVacIII). The highest IFN-γ production was detected in the rVacIII stimulated spleenocytes (2,957 pg/ml) compared with control group (845 pg/ml; $P < 0.05$) (Figure 4). However, IFN- γ production in the MTB 8.4 peptide stimulated splenocytes was low (98 pg/ml) but statistically significant compared with its control group (83 pg/ml; $P < 0.05$).

Th2-type immune response was measured based on IL-4 production detected in splenocytes culture supernatant stimulated with MTB 8.4 peptides and rVacIII protein (Figure 5). For splenocytes stimulated with rVacIII protein, there was significant increased of IL-4 production (16 pg/ml) compared with the control group (14 pg/ml; $P < 0.05$). IL-4 secretion for rSTvacIII vaccinated mice was only 1.1 fold higher than control mice compared with about 3.5 fold differences in IFN- γ production, when compared with control mice. In addition, no significant

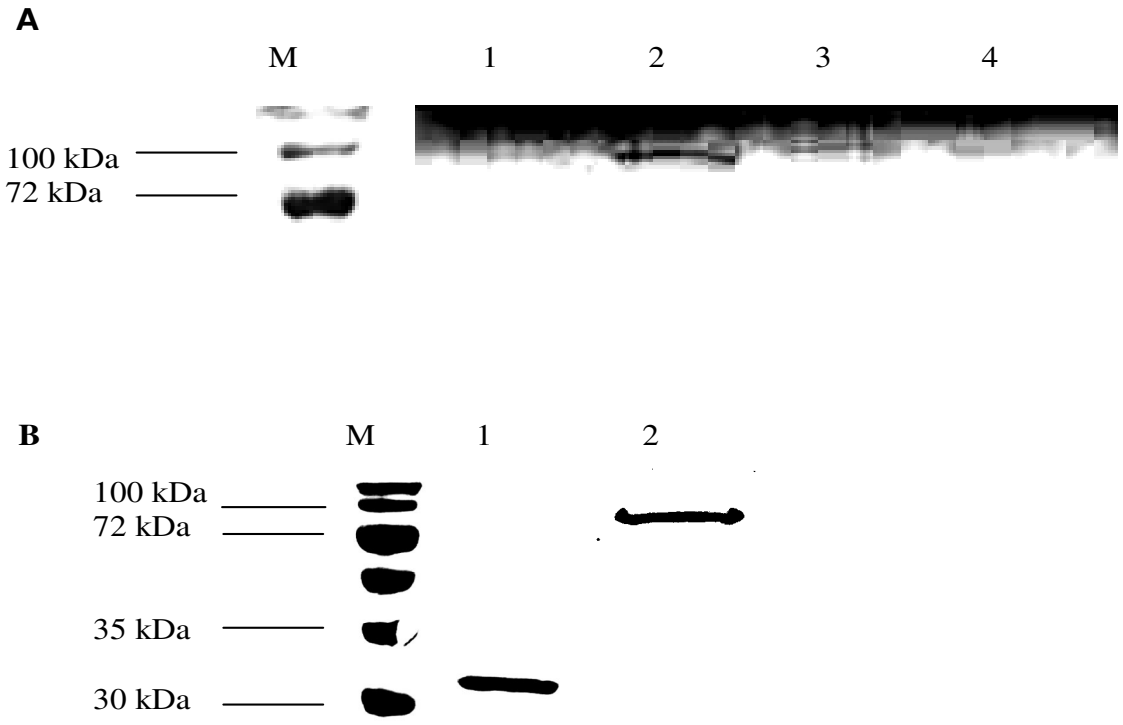

Figure 3. The western blot analyses are showing the expression of recombinant PgsA-VacIII protein by *S. typhi* Ty21a cells. (A) Pellet from cell lysate of rST from 23 h culture (negative control)(lane 1) and; pellet from cell lysate of rSTvacIII from 16 h (lane 2), 18 h (lane 3) and 23 h (lane 4) culture; (B) purified recombinant VacIII protein (Lane 1) as control positive and surface protein of rSTvacIII (lane 2). The VacIII protein was detected using HYB7-8 (monoclonal antibody against ESAT6 protein). Prestained protein ladder was used as molecular weight marker (M).

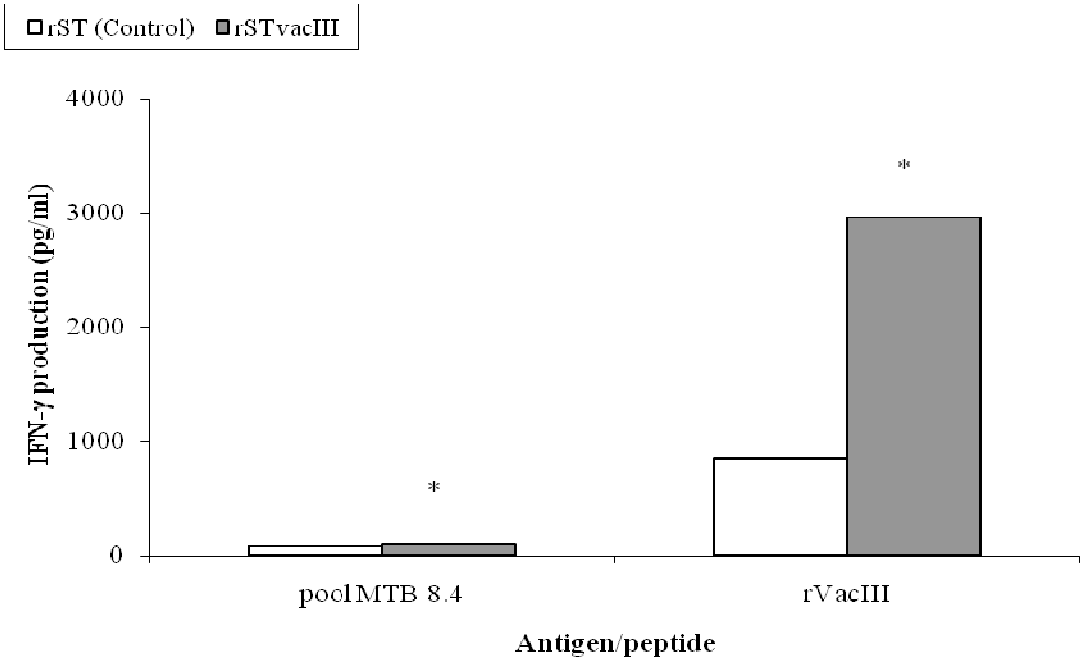

Figure 4. The concentration of IFN-γ production from the culture supernatant of splenocytes of mice vaccinated with rST (white box) or rSTvacIII (grey box). Each bar represented the mean of triplicate values. Error bars represented were of standard deviations of the means (*P < 0.05).

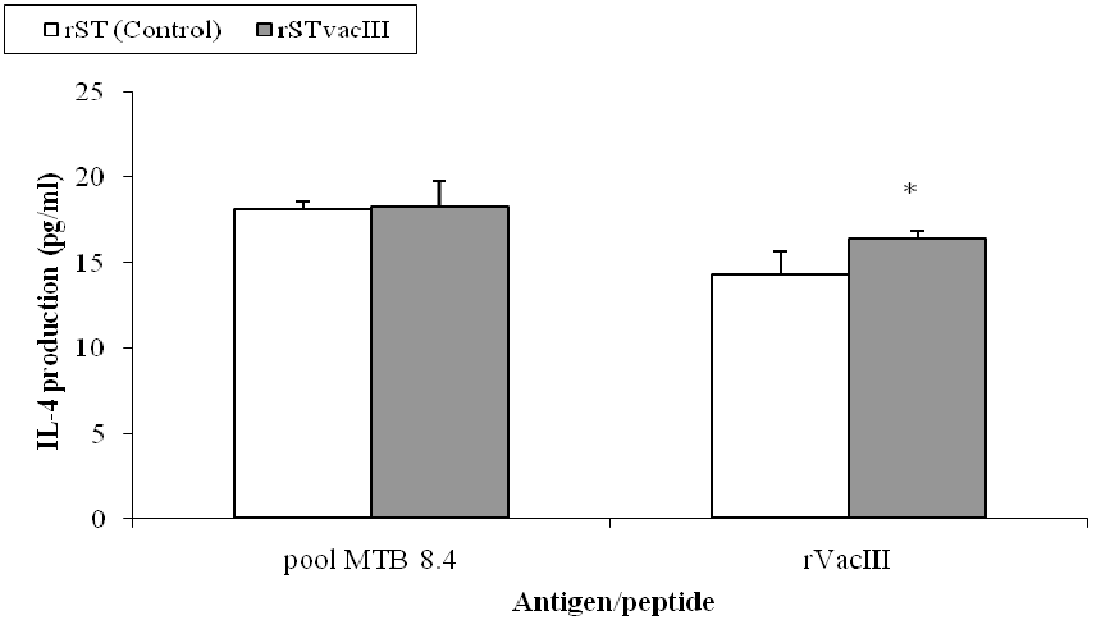

Figure 5. The concentration of IL-4 production from the culture supernatant of splenocytes of mice vaccinated with rST (white box) or rSTvacIII (grey box). Each bar represented the mean of triplicate values. Error bars represented were of standard deviations of the means (*P < 0.05).

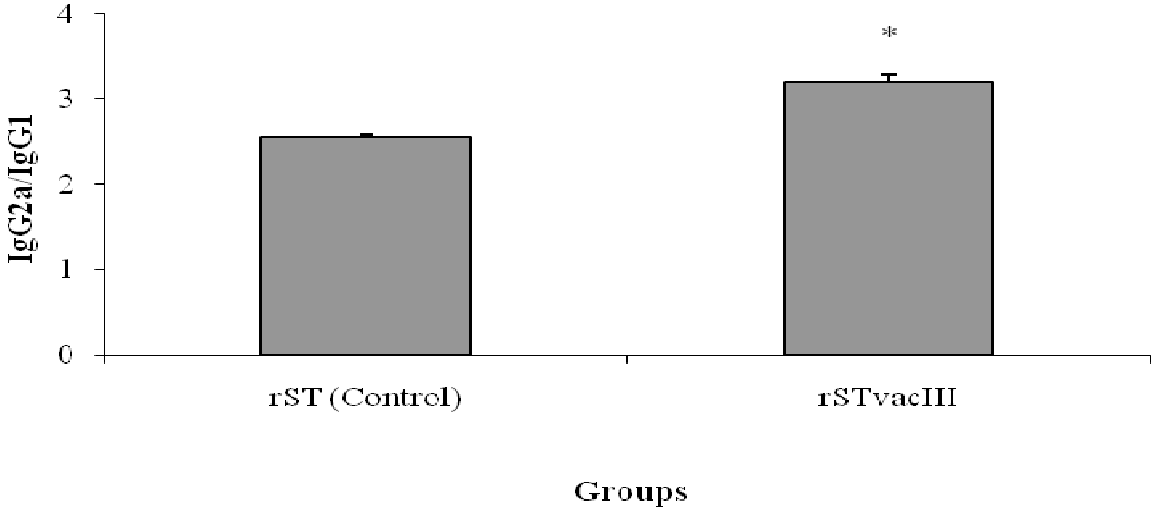

Figure 6. The value of IgG2a to IgG1 ratio of sera collected from mice vaccinated with rST and rSTvacIII. Each bar represents the mean of triplicate values. Error bars was represent of standard deviations of the means. (*P < 0.05).

increase of IL-4 production in the MTB 8.4 peptides stimulation (18 pg/ml) compared with the control group (18 pg/ml).

Antibody isotyping analysis of rSTvacIII vaccinated mice sera showed higher value of IgG2a/IgG1 ratio compared with the control group (P < 0.05) (Figure 6) which indicated that, oral vaccination of rSTvacIII was biased more towards the Th1-type immune response.

DISCUSSION

Recent progress in the fields of genomics, proteomics, molecular immunology and bioinformatics have encouraged antigenic MTB antigen discovery, resulting in the identification of a large number of MTB antigens and epitopes for TB vaccine development. These MTB antigens have been well studied and characterized

including their genes sequences which lead to the development of a various types of TB candidate vaccines including DNA vaccine.

Our group has previously constructed a DNA vaccine, designated as pVaxVacIII, which consists of several MTB epitopes. The pVaxVacIII contains the VacIII gene which was constructed using the assembly PCR technique. VacIII combines a ubiquitin moiety (UbGR) and several T-cell epitopes of mycobacterial proteins genes: ESAT6 (P1), ESAT6 (P2), PhoS1 (38kD)(129-137), PhoS1 (38kD)(65-83), PhoS1 (P3), Hsp 16.3 and MTB 8.4 genes (Fang et al., 2006; Fang et al., 2007). Based on the immunogenicity study in mice, pVaxVacIII stimulate strong Th1 cell response as characterized by high IFN-γ production and low IL-4 production (Fang et al., 2007).

In the present study, *S. typhi* Ty21a has been chosen as a vector to express the VacIII gene. The vaccine strain is selected for this study because it has been proven safe, can be delivered via the oral route and has the ability to induce MHC Class-I and Class-II immune responses in hosts (Kochi et al., 2003). Previous reports have shown that, *S. typhi* Ty21a is able to stimulate vigorous Th1 responses as indicated by the production of IFN-γ but not IL-10 or TGF-ß in human subjects (Salerno-Goncalves et al., 2003). This type of immune response is also essential for protective immunity against MTB (Brant et al., 1996; Andersen and Doherty, 2005).

Therefore, *S. typhi* Ty21a is can potentially be developed further as a vaccine against TB. In addition, the mode of delivery of *S. typhi* Ty21a which is by the oral route may result in increased compliance, safety and ease of administration (Cotton and Hohmann, 2004).

In order to enhance the efficacy of the candidate vaccine, we have used surface display protein expression technology to display the VacIII protein on the surface of *S. typhi* Ty21a. Expressing heterologous protein on the surface of attenuated *Salmonella* vector is a strategy to enhance host immune responses against the antigen (Lee et al., 2003; Lee et al., 2000). As discussed earlier in the introduction, displaying a heterologous antigenic protein on the surface of bacteria (bacterial surface display) may facilitate antigen recognition by host immune system. In order to attach the antigenic protein on the surface of bacteria, the antigenic protein should be fused with a carrier protein (anchoring motif), which generally is a protein that naturally expressed on the surface of bacteria.

Previously, our group has developed a recombinant *S. typhi* Ty21a displaying VacIII protein using ice nucleation protein (INP) as carrier protein under control of the *tac* promoter designated as *StV3* (Fang et al., 2007). This expression system requires specific conditions to express the recombinant protein namely IPTG induction and an incubation temperature of 25℃ for optimal expression (Lee et al., 2000; Fang et al., 2006). Since the oral route is used, the vaccine candidate will be introduced into an unfavorable environment for antigenic protein expression after ingestion. Even though the optimum protein expression can be achieved before and during the immunization, the protein expression may decrease after the ingestion because the environment temperature has increase from 25 to 37℃. Furthermore IPTG is very costly (Vanz et al., 2008) and may create a safety problem as the existence of chemical inducers in the purified protein product is potentially toxic to human (Poo et al., 2002; Donovan et al., 1996). Accordingly, we decided to explore the use of another surface display expression system as a means to increase the immunogenicity of antigenic protein in a more convenient expression system for oral vaccination.

The HCE-PgsA surface display expression system consists of a highly constitutive expression (HCE) promoter and the PgsA anchoring motif genes which have the ability to express recombinant protein on the surface of bacteria. The PgsA carrier protein is a poly-γ-glutamic acid (PGA) derived from *Bacillus subtilis chungkookjang,* whereas the HCE promoter is a highly constitutive expression promoter derived from *Geobacillus toebi* (Poo et al., 2002; Narita et al., 2006).

This HCE-PgsA surface display expression system has been shown useful in the area of protein expression as well as vaccine development. Narita et al. (2006) has shown that, HCE-PgsA system is able to display a 78 kDa protein on *E. coli* cells in the active form. The author also claims that PgsA protein is the first carrier protein that is able to display such a huge enzyme in conformationally active form on the cell surface of *E. coli*. Other studies have shown that, PgsA protein is also able to display the 81.5 kDa α-amylase enzyme of *Streptococcus bovis* on the surface of lactic acid bacteria (Poo et al., 2006; Lee et al 2006). This excellent capability of the PgsA protein suggests that, the HCE-PgsA system is relevant to a wide range of biotechnological applications.

In vaccine development, the HCE-PgsA expression system has been used to construct candidate vaccines against tumor and influenza virus and produced promising results. Poo et al. (2006) has demonstrated that, oral administration of *Lactobacillus casei (L. casei)* displaying human papillomavirus type 16 E7 antigen (HPV16 E7) using HCE-PgsA system stimulated cellular immune responses and anti-tumor effects in mice. Furthermore, Lee et al. has shown that, mucosal immunization of *L. casei* displaying the severe acute respiratory syndrome-coronavirus associated with S antigen (SARS-CoV S) using the PgsA system induces both systemic and mucosal immune responses against the antigen (Lee et al., 2006).

However, thus, far no candidate vaccine against TB has been developed using the HCE-PgsA system. In addition, from our review the expression system have not yet been tested in *S. typhi* Ty21a. Accordingly, it would be useful to determine if the system is capable to effectively express the VacIII protein in *S. typhi* Ty21a.

Therefore, in the present study, the HCE-pgsA system was selected for construction of the recombinant surface display recombinant *S. typhi* Ty21a, rSTvacIII, which is able to display VacIII on its surface.

The cloning process was started to construct the plasmid (pT(II)(*Nde*I)-PgsA-VacIII) which will be the plasmid for expressing VacIII on the surface of the host strain. To achieve this, the VacIII gene must be inserted into pT(II)(*Nde*I)-pgsA-HBV by substituting the HBV gene with the VacIII gene. However, there were no compatible RE sites between VacIII gene and pT(II)(*Nde*I)-pgsA.

Accordingly, a strategy was derived in which the VacIII gene was first subcloned and inserted into the pCR®2.1 TOPO® vector and then excised in such a way so as to acquire the necessary RE sites and in the correct orientation.

To do this, pTInakVacIII was digested with *Kpn*I-*Hin*dIII to obtain the VacIII gene fragment and the fragment was ligated into pCR®2.1 TOPO® vector digested with *Kpn*I-*Hin*dIII to generate pTOPO-VacIII. This resulted in the positioning of a *Bam*HI and a *Hin*dIII site just upstream and downstream of the VacIII gene, respectively, which then be used for cloning into the *Bam*HI and a *Hin*dIII sites in pT(II)(*Nde*I)-pgsA.

Then, pTOPO-VacIII was digested with *Bam*HI-*Hin*dIII to produce VacIII DNA fragment with *Bam*HI-*Hin*dIII cloning terminals. The VacIII DNA fragment was ligated into pT(II)(*Nde*I)-pgsA-HBV digested with *Bam*HI-*Hin*dIII (which removes the HBV gene) to generate pT(II)(*Nde*I)-PgsA-VacIII.

The expression of VacIII protein on rSTvacIII was confirmed by western blot. The expression of PgsA-VacIII protein under the control of HCE promoter was monitored after culturing rSTvacIII for 16, 19 and 23 h.

The western blot analysis showed that, the highest expression of PgsA-VacIII protein was found in the 16 h rSTvacIII culture followed by 19 h and the lowest was at 23 h. This result however, is not in agreement with a study done by Poo et al. (2002). The group found that, HCE promoter had the highest expression of recombinant protein after a 23 h incubation using *E. coli* BL21 as the host (Poo et al., 2002). In our study however, the expression of PgsA-VacIII protein gradually decreased after 16 h of incubation. However, such variance may be due to the protein to protein differences in characteristics and their effects on the host strains.

It should be noted that, in this study, *S. typhi* Ty21a was used as the host and the regulation of the HCE promoter in this bacteria may not be the same as in the *E. coli* host. Poo et al. (2006) also found that, *L. casei* displaying (HPV16 E7) using the HCE-PgsA system also produced low recombinant protein expression (Poo et al., 2006). Thus, the efficiency of the HCE promoter appears to host dependent.

After the expression of VacIII protein on the surface of rSTvacIII is confirmed, the candidate vaccine was subjected to immunogenicity studies. The immunogenicity studies generally involve the evaluation of specific antibody of sera from vaccinated mice and *in vitro* cytokine production by splenocytes. Both tests were performed using ELISA-based assays.

In vitro cytokines production of Th1 or Th2 cytokines is a good indicator of the type of immune responses generated after vaccination. The high production of Th1 cytokines such as IFN-γ and IL-2 are associated with cellular immune responses, while the high production of Th2 cytokines such as IL-4 and IL-10 are linked to antibody responses.

Following the homologous rSTvacIII vaccination, the results showed that, rSTvacIII vaccination generated a mixed Th1 and Th2 type immune response (Figures 4 and 5). The immune response however, was more biased towards the Th1 type responses based on the higher IFN-γ production compared with the IL-4 production.

This result was in line with the study done by our group, Fang et al. (2007), in which recombinant *S. typhi* Ty21a displaying the VacIII gene using INP as the anchoring motif under control of a *tac* promoter was used. This study showed that, mice vaccinated with the recombinant *S. typhi* Ty21a generated a mixed Th1 and Th2 type immune response (Fang et al., 2007). The result also indicates that, the immune responses generated by recombinant *S. typhi* Ty21a only depend on the immunogenicity of the antigen expressed and not by the expression system used.

The antibody isotyping results showed that, rSTvacIII have a higher IgG2a/IgG1 ratio compared with the control group. This result supported the suggestion that, the immune responses induced by rSTvacIII although mixed, were more biased towards the Th1 type immune responses.

Conclusion

In summary, we have successfully constructed a surface display *S. typhi* Ty21a expressing multi-epitope mycobacterial antigen as a candidate vaccine against TB. The immunogenicity result revealed that, the recombinant *S. typhi* Ty21a was immunogenic and can potentially be developed further as an oral vaccine against TB.

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