Cloning, protein expression and display of synthetic multi-epitope mycobacterial antigens on Salmonella typhi Ty21a cell surface

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Expressing proteins of interest as fusion to proteins of bacterial envelope is a powerful technique for biotechnological and medical applications. The synthetic gene (VacII) encoding for T-cell epitopes of selected genes of Mycobacterium tuberculosis namely, ESAT6, MTP40, 38 kDa, and MPT64 was fused with N-terminus of Pseudomonas syringae ice nucleation protein (INP) outer membrane protein. The fused genes were cloned into a bacterial expression vector pKK223-3. The recombinant protein was purified by Ni-NAT column. VacII gene was displayed on the cell surface of Salmonella typhi Ty21a using N-terminal region of ice nucleation proteins (INP) as an anchoring motif. Glycine method confirmed that VacII was anchored on the cell surface. Western blot analysis further identified the synthesis of INP derivatives containing the N-terminal domain INP- VacII fusion protein of the expected size (52 kDa).

Keywords: Ice nucleation protein, Mycobacterium tuberculosis, Surface display, Ty21a

Concept of using recombinant microorganisms for surface display of heterologous gene products has gained increasing attention in biotechnology and medicine during the last decade1,2. Displaying single or multiple epitopes, as well as complete proteins, on the bacterial surface can for instance yield recombinant vaccines that can probably be taken orally3. Surface display consists in genetically fusing a protein of interest to another protein that will permit its presentation at the surface of an organism, expressing proteins of interest as fusions to proteins of the bacterial envelope is a powerful technique with many biotechnological and medical applications4,5. Establishing expression systems to display heterologous peptides on the surface of microorganisms gained interest since this type of research holds great promise for a considerable range of applications in immunology, applied microbiology and biotechnology, especially for vaccine development.

Several anchor proteins that mediate translocation of passenger proteins through cytoplasmic and outer membranes of E. coli and their exposure on the cell surface have been used6. Several fusion protein strategies for display of relatively short peptides (less than approximately 60 amino acid residues) have been successfully displayed on the cell surface2,7. In gram-negative bacterial surface display systems, heterologous proteins were displayed on the cell surface, by insertion into surface-exposed loops of fimbrial proteins8,9 or fused to the surface exposed termini of the outer membrane proteins like LamB10 or PhoE11. Larger passenger domains could be presented on E. coli cell surface by insertion into a surface-exposed domain of E. coli flagellar proteins12 or by carboxy-terminal fusion to E. coli lipoproteins Lpp and OmpA13,14. Other types of proteins, like ice nucleation protein of Pseudomonas syringae have been used as a surface display system to display several proteins including levansucrase15, carboxymethylase16, salmobin17 and hepatitis B antigen18, chitinase 92 (Chi92) from Aeromonas hydrophila19, thermostable lipase (TliA) from Pseudomonas fluorescenc20, rat NADPH-cytochrome P450 oxidoreductase21, heme-and diflavin-containing oxidoreductase, P450 BM3 (a single, 119-kDa polypeptide)22. The synthetic gene for N-terminus of ice nucleation protein of Pseudomonas syringae was constructed using assembly PCR23.

VacII gene is a synthetic gene encoding for T-cell epitopes of selected genes of Mycobacterium tuberculosis, ESAT6, MTP40, 38 kDa, and MPT64, was constructed by assembly PCR according to Stemmer et al.,24 and cloned into eukaryotic...
expression vector pJW4303 (contains a CMV promoter that drives the transcription of the encoded genes) to create a recombinant plasmid designated as pJWVacII (Fig. 1A). The sequence of this gene has been shown in Fig. 1B. This plasmid was used in the current project as the source of VacII synthetic gene. In this study, an attempt was made to demonstrate the development of a cell surface display system by using N-terminus of ice nucleation protein from *Pseudomonas syringae* for displaying selected T cell epitopes of several *M. tuberculosis* antigens on the surface of *S. typhi* Ty21a (Ty21a).

![Diagram of pJWVacII gene](image)

**Fig. 1**—(A) pJWVacII gene; (B) complete sequence of the VacII gene aligned with the a.a. VacII amino acid sequence [Epitopes: ESAT 6 (P1, P2), MTP40 (P7), 38KDa (A, B), MPT64 (P17, P19, P20)].
Materials and Methods

VacII gene synthesis—Assembly PCR method\textsuperscript{24} was used to assemble four T cell epitopes of *Mycobacterium tuberculosis*, ESAT6, MTP40, 38 kDa, and MPT64 (GenBank accession No.: GI: 61223745; GI: 280329; GI: 129538 and GI: 1404375 respectively) into one gene designated as VacII. Briefly, equal volumes of 34 overlapping oligos were mixed together in a tube and this mixture was then further diluted 10-fold. One microliter of the pooled oligos was added to 25 µl PCR mixture contains: 2.5 µl 10× PCR buffer; 2.5 µl (0.2 mM) dNTP mixture; 4 µl (2.5 mM) MgCl2; 1U of Taq polymerase. The PCR reaction was carried out in a 0.2 ml sterile thin-walled PCR tube that began with a denaturation step at 95°C for 1min. This step was followed by 55 cycles at 95°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min followed by one cycle at 72°C for 5 min.

To amplify the desired full-length product, the annealed template (first PCR product) was diluted 10-fold in 50 µl complete PCR mix with the forward primer: 5' GTT TCG GCT AGC AGA GCA GAG CAG 3' containing a *Nhe I* site (underlined) and 5' GAT GGA TCC TTA CGG ATT GCC ATT TGG 3' containing a *BamHI* site (underlined) to allow cloning into pJW4303 expression vector. PCR reaction was performed as follows: One cycle at 95°C for 1 min; Thirty two cycle at 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min and one cycle 72°C for 5 min on a Model 9600 Thermal Cycler (Perkin-Elmer, USA). pJWVacII was constructed using rapid ligation kit (Roche, Germany) after digestion of the PCR product and the pJW4303 with *Nhe I* and *BamHI* restriction enzymes (Fig. 1A). The VacII gene sequence has been shown in Fig. 1B.

DNA manipulation—DNA manipulations were carried out by using the procedures described by Sambrook and Russel\textsuperscript{25}. To create 6His linker, 0.82 kb fragment of VocII gene was amplified from the previously constructed plasmid, pJWVacII, by performing overlapping PCR with the following primers: Forward primer [5' CTC GGG ATC CAT GAC AGA GCA GCA G 3'] and reverse primers: R1 [5' ACC TCG CAT CGG ATT GCC ATT TGG ATT 3']; R2, [5' GAT GAG AAC CTC GGA TCG GAT TGG C 3']; R3 [5' ATG ATG GTG GTG ATG AGA ACC TCG CAT CGG ATT GCC 3'] and R4 [5' CTA TAA GCT TTT AAT GAT GGT GGT GAT G 3'], and then PCR products from the amplification step were cloned into pTZ57R T/A vector according to the manufacturer instructions. All primers were manufactured by Biobasic Inc. (Canada). Polymerase chain reactions (PCRs) were carried out in 50 µl reaction buffer using 5 µl of each forward and reverse primers (0.5 mM) and 0.2 µl of High Fidelity DNA polymerase (5 U/µl). Samples were subjected to 30 cycles (at 95°, 62° and 72°C for 30 sec each) on a Model 9600 Thermal Cycler (Perkin-Elmer, USA).

Construction of fusion gene—To construct a plasmid containing a fusion of Iice nucleation protein N terminus Inak-n and VacII genes (from previous studies)\textsuperscript{23,26}, the VacII gene was obtained from pTZVacII vector by digestion with *BamHI* and *HindIII* resulting in the generation of a *BamHI-VacII-HindIII* DNA fragment. Similarly, pMSInak-n\textsuperscript{23} was digested with the same restriction enzymes generating *BamHI* and *HindIII* overhang sites (Fig. 2). Digestion was performed in 20 µl reaction mixtures. The whole digestion mixture was loaded onto a 0.8% agarose gel and electrophoresed at 100 V for 30 min. Appropriate DNA fragments were gel-purified using Ultrafree\textsuperscript{TM}™ DNA centrifugal filter device (Millipore, USA) as per the instructions of manufacturer.

Purified *BamHI-VacII-HindIII* DNA fragment (90 ng) was ligated with the *BamHI-HindIII* digested pMSInak-n plasmid in a ratio of 9:1 insert to vector using the Rapid Ligation kit (Roche, Germany) for 10 min. The ligation mixture (5 µl) was used to transformed 45 µl of *E. coli* of Top10 competent cells by CaCl\textsubscript{2} method\textsuperscript{27} and incubated overnight at 37°C. The construction of the fusion gene yielded the recombinant plasmid designated as pMSInak-nVacII as shown in Fig. 1.

Construction of expression plasmid—To create the final expression plasmid construct designated as pKMSInak-nVacII, both pMSInak-nVacII and the expression vector pKK223-3 were digested with EcoRI and *HindIII*. The generated EcoRI-Inak-nVacII-HindIII fragment was gel purified, and then subcloned into the EcoRI and *HindIII* sites of the expression vector pKK223-3 vector followed by transformation into *E. coli* XL1-Blue. The constructed expression vector was designated as pKMSInak-nVacII; the strategy for construction of this vector is summarized in Fig. 2.

Expression of recombinant protein—*S. typhi* Ty21a were transformed with plasmid pKMSInak-nVacII using CaCl\textsubscript{2} method as summarized Sambrook and Russel\textsuperscript{25}. Induction of expression were carried out as
Fig. 2—Construction of plasmid pKMSInaK-nVacII (6,070 bp), that contains a fusion synthetic gene encoding InaK-n surface display sequence, VacII gene and 6xHis tag domains under control of tac promoter.
described by Lee et al.\textsuperscript{18}. Expression of the fusion protein was induced by the addition of different concentrations of isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG, 0.5 and 1 mM) into the separate culture tubes at various time intervals (2, 4, and 6 h) of incubation, one ml samples were collected from each culture and the cells were harvested by centrifugation as described above and the pellets were stored at -20\(^\circ\) C until further use. \textit{S. typhi} TY21a transformed with pKK223-3 was used as negative control.

The bacterial cell pellets were then resuspended in 200 \( \mu \)l of lysis buffer [50 mM Na\textsubscript{2}HPO\textsubscript{4}; 300 mM NaCl; 10 mM, Imidazole] containing 0.5 mg/ml lysozyme and incubated on ice for 10 min. The cell lysate was disrupted by mild sonication on ice for 1 min with 20 sec pulses and 20 sec interval between pulses using an Ultrasonic processor XL with Taperd microtip probe (Misonix, Inc. USA). The sonicated solution was centrifuged at 14,000 rpm for 15 min at 4\(^\circ\)C and the pellet was resuspended in 200 \( \mu \)l of PBS and stored frozen at -20\(^\circ\) C until used for SDS and Western blot analysis.

\textit{Determination of protein concentration}—Protein concentration was determined using a Bio-Rad protein assay kit which was based on a method described by Bradford\textsuperscript{28}. The absorbance at 595 nm was measured using Lambda EZ150 UV/Vis spectrophotometer.

\textit{SDS-PAGE and immunoblotting}—Prepared protein samples from recombinant \textit{S. typhi} TY21a were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli\textsuperscript{29}, with separation gel (12.5\%) in the presence of 2-mercaptoethanol. The proteins, thus, obtained were transferred at 12V for 30 min to nitrocellulose membranes (Hybond\textsuperscript{TM}-C extra nitrocellulose membrane; Amersham, Sweden) in a Trans-Blot semi-dry transfer cell (Bio-Rad) by standard protocols\textsuperscript{25,30}. After transfer, nitrocellulose membrane was blocked with 3\% solution of skim milk powder in Tris-buffered saline solution containing Tween (TBST; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05\% Tween 20) with gentle shaking for 1 h. The blocking solution was discarded, and the membrane washed for 5 min three times in TBST. The membrane was incubated for 1 h at room temperature in TBST buffer containing Ni-NTA\textsuperscript{4P} conjugate (1/1000 dilution)\textsuperscript{31}. The membrane was washed for 10 min three times with TBST at room temperature. For colour detection, the membranes were incubated in alkaline phosphatase buffer [100 mM Tris-HCl (pH 9.5); 100 mM, NaCl; and 50 mM, MgCl\textsubscript{2}] containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP) according to the manufacture’s instructions (Qiagen, USA).

\textit{Extraction of cell surface proteins}—To confirm that Inak-nVacII protein was displayed on the cell surface of the bacteria, surface expression of the Inak-nVacII fusion protein was assessed using a standard method with 0.2 M acid glycine buffer (pH 2.2) as described by McCoy et al.\textsuperscript{32} and Lelwala-Guruge et al.\textsuperscript{33}. The protein concentration was determined by Bradford method\textsuperscript{28}.

\textit{Purification of recombinant protein by metal chelate affinity}—For evaluation of immune response, the Inak-nVacII protein containing the 6xHis tag at the C-terminus of the protein was purified from induced cultures of r-STVII using B-PER\textsuperscript{6xHis}Spin purification kit (Pierce, USA). For this experiment, cultures of r-STVII were grown at 25\(^\circ\)C to maximize the expression of the protein at surface as described by Lee et al.\textsuperscript{18}.

\textbf{Results}

To determine whether the VacII fragment was successfully ligated to the Inak-n of pMSInak-n, plasmid DNA was extracted from selected transformants which were then digested with \textit{Eco}RI and \textit{Hind}III to release the fusion gene. Agarose gel electrophoresis of the digestion reaction revealed the presence of two bands. An upper band corresponding to the linearized pCR\textsuperscript{2.1-TOPO}\textsuperscript{®} vector (about 3900 bp), and a lower band corresponding to the expected molecular size for Inak-nVacII of approximately 1500 bp (Fig. 3A, lane 3). pMSInak-nVacII was also digested with \textit{Bam}HI and \textit{Hind}III, which resulted in the release of the VacII DNA fragment as shown in Fig. 3A (lane 4), with the expected size of approximately 870 bp and another band with approximate size of 4,500 bp which is the linearized plasmid pMSInak-n.

To confirm the presence of Inak-nVacII fusion gene in pKMSInak-nVacII, restriction digestion was performed with \textit{Eco}RI and \textit{Hind}III. The agarose gel electrophoresis analysis showed the presence of a lower band corresponding to the expected size of approximately 1500 bp and a higher band representing the linearized pKK223-3 of approximate size of 4584 bp (Fig. 3B, lane 3).
The results of this study showed that the Inak-nVacII protein was successfully expressed in r-STVII as shown by the presence of a band of the expected molecular weight of approximately 52 kDa in SDS-PAGE stained with Coomassie brilliant blue (Fig. 4A, lanes 4-6). Optimum IPTG concentration was found to be 1 mM (Fig. 4A, lane 6) and optimum induction time at 25°C was 6 h. The control strain TypK before and after induction with 1 mM IPTG for 4 h, showed no bands at the expected molecular weight (Fig. 4A, lanes 2 and 3, respectively).

Expression of Inak-nVacII was confirmed by Western blot of the same samples (Fig. 3b, lanes 4-6) with the expected molecular weight at various IPTG concentrations at 25°C with an optimum expression at 1 mM for 6 h of induction. The control strain, TypK, showed no bands at the expected molecular weight (TypK; Fig. 4B, lanes 2 and 3).

The present results showed the presence of one band of the expected size on the surface protein fraction and the whole lysate fraction (Fig. 5, lanes 3 and 5 respectively), whereas the cell pellet fraction of r-STVII after extraction of the surface protein did not show any band (Fig. 5, lane 4). Similarly, protein extracted from TypK also showed no band (Fig. 5, lane 2). These results suggested that under the
Experimental conditions used, r-STVII were able to express and display the Inak-nVacII protein on its surface. The purified protein was analyzed by SDS–PAGE (12%) and the results showed that the recombinant protein was successfully expressed in r-STVII after induction by IPTG (1 mM) and purified by the method described above. The presence of the protein was easily detected in SDS–PAGE stained with Coomassie brilliant blue (Fig. 6, lanes 3 & 4). The 6xHis tagged Inak-nVacII protein was shown to have been purified to homogeneity by Ni–NTA affinity chromatography with a single band of approximately 52 kDa.

**Discussion**

Cell-surface display allows peptides and proteins to be displayed on the surface of microbial cells by fusing them with the anchoring motifs. The present study reports the development of a recombinant Ty21 strain which displays the VacII on its surface utilizing a surface display protein called ice nucleation protein (INP) originally found in *P. syringae*. The most important feature of this protein is the ability of N- or C-domains to display the protein on the surface of bacterial cells. Ability of INP from *P. syringae* to function as an anchoring protein to display heterologous protein on the surface of *E. coli* is first demonstrated by Jung *et al.* Use of INP was chosen for this study because no additional gene product was necessary for the crossing of INP through the cytoplasmic membrane and insertion into the outer membrane, besides that the INP is not processed during transport and has no typical signal peptide sequences. In this study, the N-terminus rather than the previously described whole protein of INP was used to display VacII on the surface of Ty21a. Although there are some examples of expression of peptides and small antigens on the surface of *Salmonella*, the expression of multi-epitopes mycobacterial protein on the surface of *Salmonella* has not yet been reported.

To construct the surface display expression vector, the VacII gene, constructed previously, was fused with InaK-n gene construct in this study. This was achieved through amplification of the VacII gene using specific primers containing appropriate restriction sites. Fused protein was also tagged at C-terminus with 6xHis to simplify the detection of expressed protein and to simplify purification by metal chelate chromatography. In addition, four amino acid residues met-arg-gly-ser were also added between the VacII gene and the 6xHis residues to allow the tagged protein to be fully exposed for effective binding to the resin. The 6xHis moiety has a high affinity for nickel-chelate-nitrilotriacetate (Ni-NTA) which is prepared as an agarose chromatography column. Binding of proteins with the 6xHis tag to Ni-NTA agarose is not affected by conformation of the expressed proteins and therefore, can be purified under denaturing conditions.

Expressions of recombinant proteins depend among other factors, on the promoter activity. Several protein expression systems with different promoters have been developed for effective expression of foreign proteins in *E. coli*. Among these include the vector pKK223-3, an expression vector containing a *trp-lac* (*tac*) fusion promoter and the transcription terminator *rrn* operon which improves plasmid stability. It has been observed that in *Vibrio cholerae* based heterologous antigen expression system, the *tac* promoter is better suited for generation of an *in vivo* immune response than both a Hsp promoter and an iron
regulated promoter. Lee et al., have also shown that the tac promoter is effective for heterologous expression on the cell surface of Ty21a. In this study, the recombinant protein was successfully expressed in Ty21a vaccine strains. Display of the VacII protein on the surface of Ty21a was also successful as observed by Western blots of surface extracted protein homogenate with Ni-NTAAP conjugates. The recombinant protein was also successfully purified from r-STVII using Ni-NTAAP affinity column to more than 90% purity as assessed by SDS polyacrylamide gel electrophoresis that was observed from the washing step in which the concentration of unbounded protein was reduced after washing for a few time. These results suggested that the column was completely washed and the unbounded proteins removed completely. Western blotting was performed to confirm the expression of the fusion protein. Western blotting analysis showed r-STVII protein was successfully expressed in S. typhi Ty21a cells.

Development of a multivalent mycobacterial protein r-STVII provides another alternative option in subunit vaccine development against TB. The synthetic gene called VacII encoding T-cell epitopes from four M. tuberculosis genes ESAT6, MTP40, 38 kDa, and MPT64 was chosen because this synthetic gene have been found to induce the secretion of INF-γ when immunized intramuscularly into C57BL/6 mice.

In conclusion, we developed a novel cell surface display expression vector in which P. syringae Inak-n was used as an anchoring motif. A large polypeptide up to 287 amino acids long could be successfully displayed on the S. typhi Ty21a outer membrane, which was not consistent with the size limit for peptides to be inserted as previously suggested for other fusion motifs. Furthermore, the recombinant strain expressing VacII on the cell surface developed in this study might be ideal to develop a live Tuberculosis vaccine.

References


