Original article

Expression and immunological evaluation of elongation factor Tu of *Streptococcus suis* serotype 2

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Abstract

Streptococcus suis serotype 2 (SS2) is considered as a major pathogen that causes sepsis and meningitis in piglets and humans, but knowledge of its antigenic proteins remains limited so far. The surface-related proteins of pathogens often play significant roles in bacterium-host interactions and infection. Here, we obtained the elongation factor Tu (EF-Tu) gene of *Streptococcus suis* and constructed the recombinant expression plasmid successfully. The target recombinant plasmid was then expressed in *Escherichia coli* and the immuno-protection of the recombinant protein was subsequently evaluated as well. The EF-Tu gene of *Streptococcus suis* is 1197 bp in length, encodes 398 amino acids. The target recombinant EF-Tu (rEF-Tu) protein can recognize the antiserum of *Streptococcus suis* and can provoke obvious humoral immune responses in rabbits and conferred protection to rabbits against *Streptococcus suis* ear-vein challenge, implying that the EF-Tu may be used as an attractive candidate antigen for a component of subunit vaccine.

Key words: *Streptococcus suis*, EF-Tu, prokaryotic expression, protection

Introduction

Streptococcus suis (*S. suis*) is a major pathogen that causes a wide variety of diseases in the porcine industry, that clinically features with meningitis, endocarditis, arthritis, pneumonia and septicaemia with sudden death resulting in significant economic losses worldwide (Zhang and Lu 2007, Staats et al. 1997). *Streptococcus suis* that was initially reported as an etiological agent in 1954, and subsequently frequent-ly-occurring bacterial infection (Wertheim et al. 2009, Gottschalk et al. 2007). Humans *S. suis* infections have been reported sporadically worldwide (Kay et al. 1995, Tarradas et al. 2001, Tang et al. 2006). However, two large-scale outbreaks of lethal human SS2 infections with a hallmark of streptococcal toxic shock-like syndrome (STSLS) occurred in China (Tang et al. 2006, Ye et al. 2006, Yu et al. 2006). Although many virulence factors of *S. suis* serotype 2 (SS2) have been identified, such as suilysin (SLY),

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extracellular protein factor (EF) and muramidase-released protein (MRP), they still sounds fragmentary, and cannot explain the pathologic mechanisms underlying the high pathogenicity of the bacterium (Berthelot-Herault et al. 2005).

Gram-positive pathogenic bacteria usually express many specific surface-related proteins that involved in many crucial biological events during infection, including interaction with the components of the host extra-cellular matrix (ECM), adhering to host cells, invading, and evading host defenses (Pian et al. 2012). Elongation factor-Tu (EF-Tu) is responsible for the entry of aminoacyl tRNA into a specific site of the ribosome. It is an important multifunctional protein which has been shown to participate in varieties of diseases or cellular processes, including virus replication, apoptosis, cytoskeletal organization, translational control and signal transduction (Zhang et al. 2013). EF-Tu of *S. suis* had already been confirmed as an antigenic membrane-associated protein and participated in the adhesion of SS2 to host cells. (Zhang and Lu 2007a, Li et al. 2015). Moreover, EF-Tu is a very conservative surface protein of bacteria (Zhang and Lu 2007a). We suspect that EF-Tu has great potential to become antigenic component of subunit vaccine against *S. suis*. Therefore, this study aim to study the protective efficiency of EF-Tu which may provide solid foundation for the vaccine development.

Materials and Methods

Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animal ethics committee of the Henan Institute of Science and Technology.

Bacterial strains, plasmids and sera

Streptococcus suis standard strain C55606 was bought from the China Institute of Veterinary Drug Control. SS2 strain SC22 was isolated from the diseased pigs in the Sichuan Province and saved by the Shandong Binzhou Animal Science and Veterinary Medicine Academy (Binzhou, China). The *E. coli* DH5α, *E. coli* BL21 (DE3) and pET 32a vector were saved by the same institute. The pMD18-T vector was purchased from TaKaRa (Dalian, China). The serum against SS2 was collected from pigs experimentally infected with the SS2 strain SC22 and saved by the aforementioned institute.

Reagents

All restriction endonucleases, T4 DNA ligase, protease K, PCR reagents, isopropyl β-D-1--Thiogalactopyranoside (IPTG), DNA ladder and UNIT-10 spin column DNA gel extraction kits were products of TaKaRa (Dalian, China). Plasmid extraction kits were purchased from Sangon Biotech (Shanghai, China). Bacterial genomic DNA extraction kits were purchased from BioTeke (Beijing, China). Nickel ion-agarose resin for His-tagged protein purification was purchased from CoWin Biotech (Beijing, China). NanoDrop 2000/2000C spectrophotometer was purchased from Thermo Scientific (Boston, USA), Horseradish peroxidase-labeled staphylococal protein A (HRP-PPA) was purchased from Boster (Wuhan, China). DAB substrate kit was purchased from Tiangen Biotech (Beijing, China). Other reagents, imported or domestically produced, were of analytical grade.

Propolis was purchased from Qingkun Beeswax honeycomb processing factory, Shandong province. The propolis adjuvant (PA) was prepared as previously described (Ma et al. 2011). Briefly, the propolis was ground and macerated with absolute ethanol for 10 days, agitation 10 min daily. Then, the solvent was evaporated and the resulting dried matter was dissolved in phosphate buffer solution (PBS, pH 6.2), at a final concentration of 40 mg/mL. rEF-Tu propolis subunit vaccine (0.67 mL with 400 μg of rEF-Tu) and *S. suis* C55606 propolis inactivated vaccine (8×10^9 CFU/mL, 0.67 mL) were prepared by Lvdu Veterinary Biologicals Co. Ltd. (Binzhou, China) according to NO. ZL89106871.6 patent.

Extraction of genomic DNA

The SC22 was inoculated on Todd-Hewitt broth (THB) agar plate by streak culture. A single colony was then inoculated into THB liquid medium and incubated at 37ºC for 16 h with shaking at 200 r/min. Genomic DNA was extracted using the method described by the manufacturer.

PCR amplification, cloning and sequencing of EF-Tu

According to full-length sequence of SS EF-Tu in NCBI and all of the restriction sites on the gene and pMD18-T, choose the appropriate restriction sites and add to both ends of each gene primers. The primer sequences is as follows: forward: 5'-GTGGATCCATGTCAA ATGCCAAAG-3';

reverse: 5'-GTTAGAGTCGACTTATACCAAACC TTGG-3'. The forward and reverse primers contained *Bam*H I and *Xho* I recognition sequences, respectively. The expected size of the EF-Tu was about 1 197 bp. The conditions for PCR of 32 cycles were: 40 s at 94ºC for denaturation, 45 s at 53ºC for annealing, 1 min for extension, and 10 min for the final extension at 72ºC. The resulting product was separated on agarose gel, digested and cloned into the corresponding sites in the pMD18-T vector. The resulting products were transformed into *E. coli* DH5α competent cells. Through white-blue plaque selection and restriction enzyme, a positive clone named pMD18-T-EF-Tu was identified and sequenced by Sangon Biotech (Shanghai, China).

Construction of prokaryotic expression plasmid

Recombinant plasmid pMD18-T-EF-Tu was digested with *Bam*H I and *Xho* I, and a linearized fragment encoding the EF-Tu was cloned into pET-32a vector using the similar method described above. The aforementioned products was transformed into the *E. coli* DH5 alpha competent cells, followed by resistance screening on ampicillin-containing LB agar plate. After incubation at 37ºC overnight, the single colonies were proliferated and plasmid DNA was purified. The positive transformants (named pET-32a-EF-Tu) were identified by restriction enzyme and sequenced by Sangon Biotech (Shanghai, China).

Inducing expression and purification of EF-Tu protein

The positive recombinant plasmid was transformed into *E. coli* BL21 competent cells, and the single colonies were cultured in LB liquid medium at 37ºC with shaking at 200 r/min. A_{600} of the cell culture was in the range of 0.6-1.0, IPTG was added to final concentration of 0.5 mM and the culture was further incubated at 37ºC for 5 h with shaking at 200 r /min. Cells transformed with pET-32a, the culture without IPTG and wild-type (BL21) cells were used as controls. Following centrifugation, the bacterial pellet was lysed in 2 × SDS sample buffer by boiling for 5 min. After centrifugation at 12 000 r/min for 3 min, 10 uL of supernatant was subjected to SDS-PAGE to verify expression of recombinant EF-Tu protein. Under the optimized conditions, the bacteria carrying the recombinant plasmid were proliferated and induced with IPTG. The cells were harvested by centrifugation at 7 000 r/min for 15 min. The cell pellets were then resuspended in lysis buffer and disrupted by ultrasound. Then, PMSF was added to the yielded supernatant at a final concentration of 3 mM and incubated at RT for 30 min. The purification of rEF-Tu protein was carried out using nickel ion-agarose column by following the manufacturer's protocol. The total bacterial protein and purified protein were measured using NanoDrop 2000/2000C spectrophotometer (Thermo Scientific; Boston, USA) to determine the concentration of target protein in total bacterial protein.

Immunization and sera collection

A total of 9 New Zealand rabbits (weighing 2.0-2.5 kg) (Shandong Binzhou Animal Science & Veterinary Medicine Academy) were randomly divided into three groups (three rabbits each group). Group 1 was subcutaneously vaccinated with 400 μg recombinant EF-Tu protein emulsified in propolis (which were prepared by Lvdu Veterinary Biologicals Co. Ltd. (Binzhou, China) according to NO. ZL89106871.6 patent) and boosted using emulsion with propolis at 14 days post first injection. Group 2 and 3, as control groups, received an equal volume of PBS and propolis, respectively. Blood samples were collected from the tail vein at 0, 7, 14 and 28 days post first immunization, respectively. Serum was separated by centrifugation for determination of titer via indirect ELISA. 96-well microtiter plates were coated overnight at 4ºC with 10 μg/ml rEF-Tu protein, and the subsequent operation was proceeded reference to previously described (Xia et al. 2011).

Convalescent serum preparation and western blot assay

Three 30-day-old weaning SPF minipigs were selected and fed separately. The levels of specific antibody were detected to guarantee the antibody-negative pigs by ELISA before infection. Infection was performed with $1×10^8$ CFU of S. suis strain JZLQ022 (MRP⁺ EF⁺ SLY⁺ serotype 2 strain) via the intravenous injections. The porcine rectal temperature and clinical symptoms were observed and recorded daily after challenged. After 3 weeks the pigs were sacrificed, serum was collected and stored at -20ºC until utilisation (Qu et al 2016).

The purified rEF-Tu proteins were separated via SDS-PAGE, then transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% (w/v) BSA at room temperature for 4 h. Subsequently,
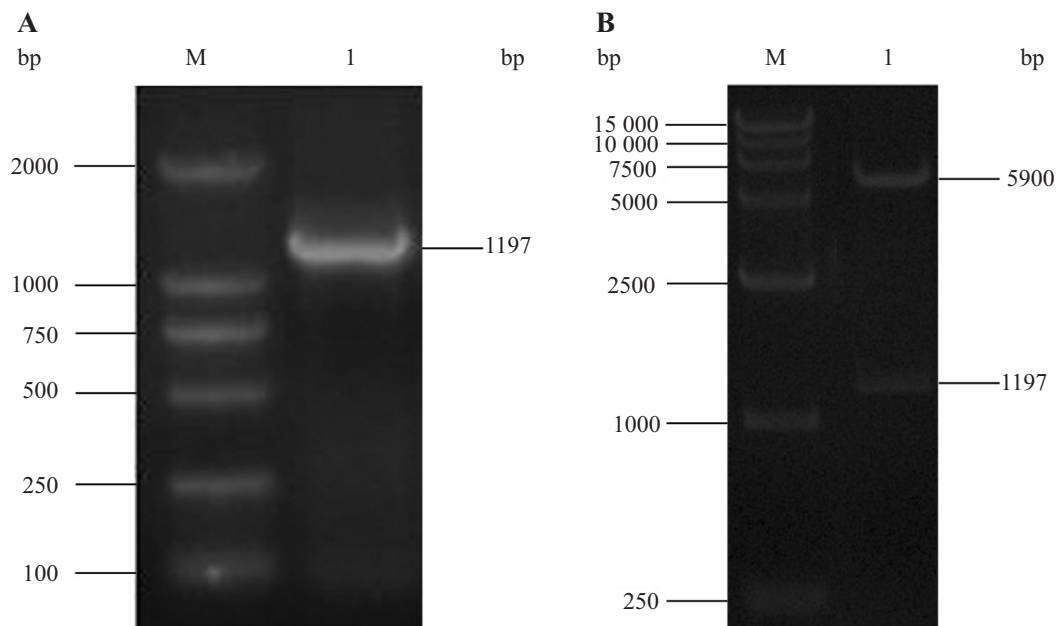
A

B

Fig. 1. Cloning of EF-Tu gene and identification of pET-32a-EF-Tu. A. Products of EF-Tu gene by PCR. M. DL2000 Marker; 1. products of EF-Tu gene by PCR. B. Identification of pET-32a-EF-Tu digested with *Xho*l I/BamH I. M. DL5000 Marker; 1. pET32a-EF-Tu/*Xho*l I+*Bam*H I.

the membranes were incubated with convalescent serum from *S.suis*-infected pigs overnight at 4ºC. The membranes were washed five times (10 min per wash) in TBST and then incubated with HRP-PPA for 1 h at 37ºC. Finally, DAB substrate was used for color development.

The immunoprotective effect of the rEF-Tu protein in rabbits

To determine the minimum lethal dose (MLD) of *S. suis* standard strain C55606, a total of 28 New Zealand white rabbits were selected and randomly assigned to seven groups. Each group of four rabbits was challenged with 1 mL of cultures containing 3000, 2500, 2000, 1500, 1 000, 500 CFU of *S. suis* standard strain C55606 via the ear vein, respectively. 1 mL of sterile modified martin medium served as control.

A total of 40 New Zealand white rabbits were selected and randomly assigned to four groups. Each group of ten rabbits was immunized with rEF-Tu propolis subunit vaccine (0.67 mL with 400 µg rEF-Tu protein), *S. suis* C55606 propolis inactivated vaccine (8×10^9 CFU/mL, 0.67 mL), rEF-Tu, sterile modified martin medium via subcutaneous injection, respectively. After fundamental immunity 14 days the rabbits were given an intensive vaccination with the same dose and approach as the first time. Ten days after booster immunization, the rabbits are challenged with a MLD of *S. suis* standard strain C55606. The mortality of rabbits was observed for 7 days post-challenge.

Results

The cloning of SS2 EF-Tu gene and construction of it's expression vectors

Using the genomic DNA extracted from the strain SC22 as templates, an approximately 1200-bp target band appeared on the agarose gel after amplification with the EF-Tu-specific primers (Fig. 1A). The sequencing results revealed that the complete ORF of EF-Tu gene was 1197 bp, encoding 398 amino acid residues. As analyzed using NCBI Blast, the obtained EF-Tu sequence had nucleotide similarity of 100% to the partial sequence coding of amino acids in protein (CDS) of EF-Tu gene sequence of *SS2* China Vaccine Strain HA9801.

A 1197-bp target fragment and a 5 900-bp vector fragment appeared after the recombinant plasmid pET-32a-EF-Tu was digested with *Xho* I and *Bam*H I, as shown by the electrophoresis (Fig. 1B). The sequencing results of the positive recombinant plasmid demonstrated the successful construction of the prokaryotic expression plasmid (pET-32a-EF-Tu).

Expression and purification of rEF-Tu

After the *E. coli* BL21 (DE3) carrying the pET-32a-EF-Tu plasmid was induced by IPTG, an approximately 62.3 kDa protein band appeared as evidenced by SDS-PAGE electrophoresis. As shown
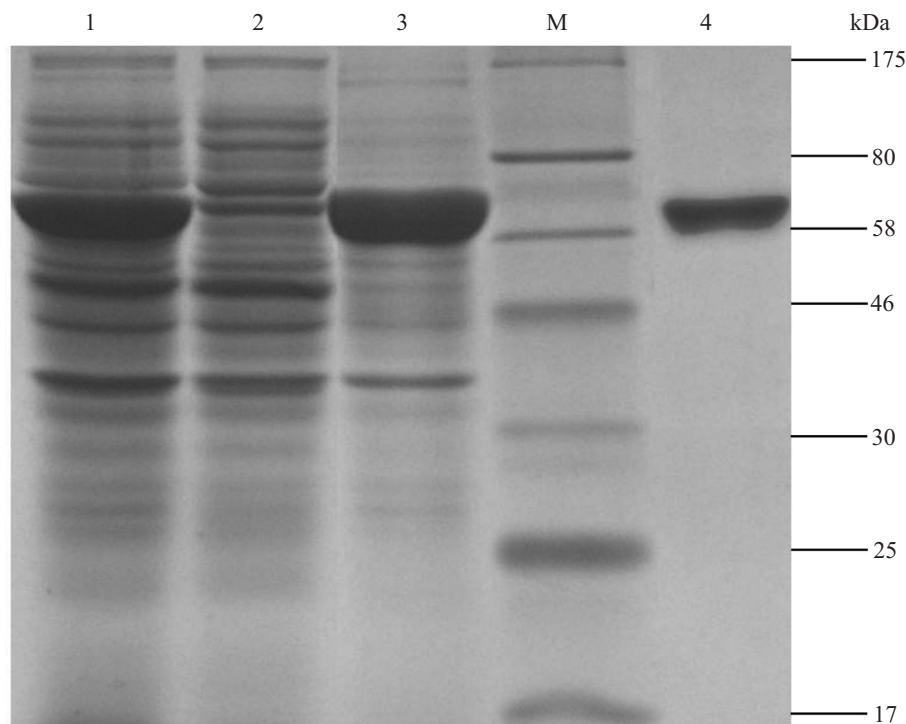
Fig. 2. SDS-PAGE analysis of pET-32a(+)-EF-Tu fusion protein. M. Protein marker; 1. pET-32a(+)-EF-Tu transformants With IPTG induction; 2. Insoluble protein of BL21/pET-32a(+)-EF-Tu transformants; 3. Sediments protein of pET-32a(+)-EF-Tu transformants; 4. Purification of sediments protein of pET-32a(+)-EF-Tu transformants.

by the SDS-PAGE, a band with MW of around 62 kDa appeared in both the supernatant and precipitate at 37°C for 6 h with shaking at 200 r/min after adding a concentration of 0.5 mM IPTG (Fig. 2). The protein bands were analyzed using Alpha VIEW software, and the results indicated that the EF-Tu fusion protein was successfully expressed. Furthermore, highly pure recombinant protein was obtained using nickel ion-agarose affinity chromatography according to the instructions (Fig. 2).

Immunogenicity and immunoreactivity of rEF-Tu protein

The mean antibody titers to the rEF-Tu protein in the New Zealand rabbits were detected by indirect ELISA. As shown in Fig. 3A, antibody levels of rabbits from immunized group were much higher than those of two control groups (PBS, propolis). All immunized rabbits generated remarkable antibody responses compared to both controls after the first immunization. After the second immunization, the mean titers of antibody in immunized rabbits gradually increased. Two weeks after the second immunization, antibody level in immunized group appeared a slight decrease, but maintained a consistent level of antibodies which indicated that rEF-Tu-immunized rabbits had arrived with a remarkable antibody level. As

shown in Fig. 3B, the rEF-Tu protein could react with the serum against *SS2* collected from the experimentally infected pigs, as demonstrated by a band at 62.3 kDa in western blotting analysis (Fig. 3B).

Immunoprotection of rEF-Tu protein in rabbits

We tentatively determined that MLD of *S. suis* standard strain C55606 was 1 500 CFU/rabbit and cause death within the first three days. One day post-challenge, we observed that the only one rabbit died in the EF-Tu propolis subunit vaccine group. Three rabbits died in the subsequent 2 days. Another seven rabbits experienced mild symptomatic *S. suis* infection and began to recover, and survived until the end of the experiment. *Streptococcus suis* C55606 propolis inactivated vaccine group and EF-Tu subunit protein group survival rates were 70% and 50%, respectively. Ten rabbits were all died within 3 days in the control group. These findings suggested that rEF-Tu protein can confer some immuno-protection in rabbits against virulent SS2 infections (Fig. 4).

Discussion

SS2 poses a serious threat to porcine industry and public health; deeper insights into the pathogenic
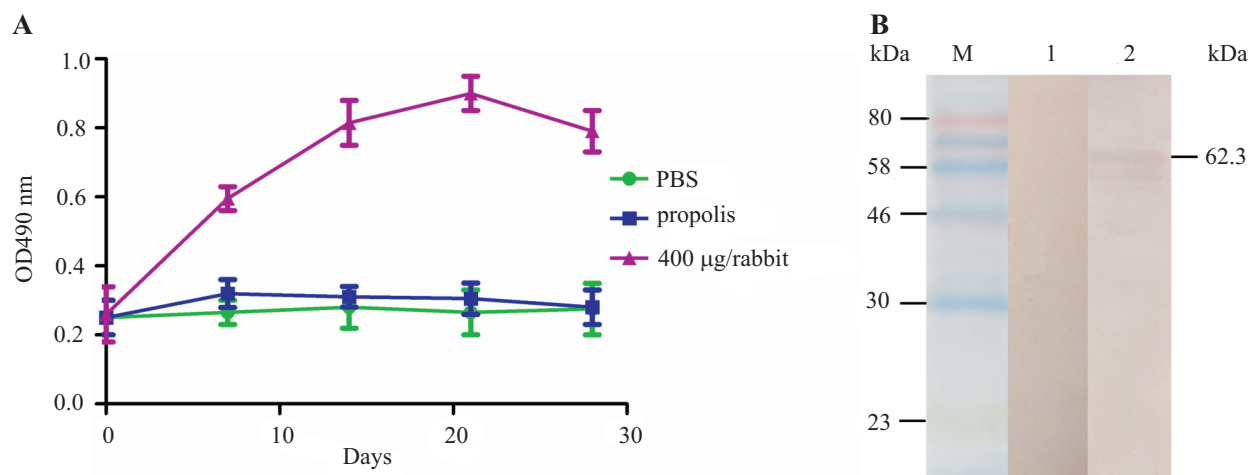
A

B

Fig. 3. Immunogenicity and immunoreactivity of rEF-Tu protein. A. Detection of antibody titers in serums from rabbits immunized with rEF-Tu, propolis and PBS. Data points shown the mean OD490 value. B. Western blot analysis of rEF-Tu protein using antiserum of SS2. M. protein marker; 1. The purified product of pET-32a-EF-Tu did not react with serums from pre-infected swine; 2. The purified product of pET-32a-EF-Tu interact with experimental infected swine serums.
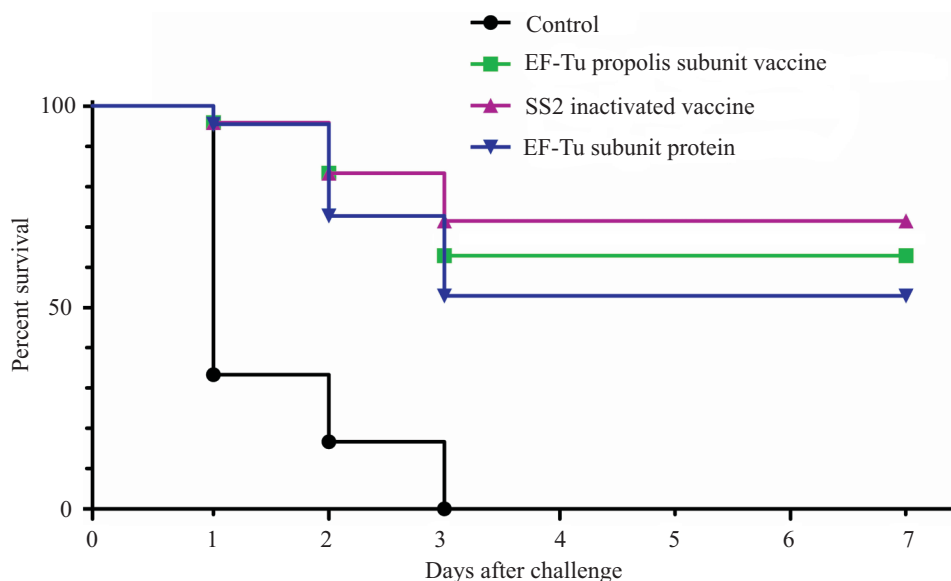

Fig. 4. Protective efficiency of rabbits immunized with rEF-Tu protein.

mechanism and development of safe and efficient vaccine are the useful strategy to combat against the bacterium infection. *Streptococcus suis* express varieties of surface-related proteins, the majority of which determine their virulence and participate in pathogen-host interactions (Pian et al. 2012). Bacterial surface-related proteins are likely to interact with the host immune system and are components of effective vaccines (Rodriguez-Ortega et al. 2006, Zhang and Lu 2007). After more than 30 years of research, there are no less than 15 protective antigens identified, such as MRP, SLY, GDH and enolase, exhibit favorable immune protection (Feng et al. 2014). However, there is

no proven and commercially available subunit vaccine for use in swine to fight against the infection caused by *S. suis* (Fittipaldi et al. 2012). Thus, the other antigenic components in SS2 need to be further studied for subunit vaccine development. In the present study, the recombinant expression vector pET-32a-EF-Tu was successfully constructed, and the rEF-Tu protein was efficiently expressed. 60% of model animals immunized by the rEF-Tu protein with propolis as adjuvant were protected after injection of SS2 as a virulent pathogen.

Many studies have demonstrated that EF-Tu is a conserved-multifunctional bacterial protein and is

an important part of the bacterial membrane cytoskeleton (Mayer 2003, Jonak 2007). A recent study indicate that EF-Tu may play a formerly under-appreciated role as a bacterial virulence factor and have an evident effect to promote early colonization of host cells of *Pseudomonas aeruginosa* (Barbier et al. 2013). Whitney et al. report the unexpected requirement for EF-Tu in Tse6 toxicity which disrupts the core metabolism of recipient cells (Cabeen and Losick 2015). Studies have demonstrated EF-Tu as an important immunodominant protein for antigen discovery against *mycoplasma* and other intracellular bacterial pathogens (Bunk et al. 2008, Gupta et al. 2009 Nieves et al. 2010, Xu et al. 2012). Furthermore, immunoproteomics studies found that EF-Tu from *S. suis* exhibited specific binding activity to both human LN and human FN, and the EF-Tu-LN or EF-Tu-FN interaction played a role in bacterial colonization (Li et al. 2015). Taken together, all these findings support our observation of immunogenic EF-Tu in the membrane of *S. suis*. In this study, western blotting showed that the rEF-Tu protein of SC22 can react with polyclonal serum of *S. suis*. Rabbit immunization confirmed the immunogenicity of rEF-Tu protein. Furthermore, rEF-Tu protein can confer some immuno-protection in rabbits against virulent SS2 infections. These results suggest that the EF-Tu is an important antigenic protein of *S. suis* and may become potential vaccine candidate.

The serotypes of *S. suis* are numerous, cross-protection among each serotype is in a low level, there is a great deal of difficulties in vaccination with traditional vaccines. Here, we confirmed that rEF-Tu protein can confer some immuno-protection in rabbits against virulent SS2 infections. In subsequent experiments, we further extend protection to cover additional pathogenic serotypes, including SS1, SS7 and SS9, to verify the feasibility of EF-Tu protein as a universal vaccine.

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