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RESEARCH ARTICLE

Characterization of Elongation Factor Tu of *Mycoplasma ovipneumoniae*

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ABSTRACT

Mycoplasma ovipneumoniae is considered as an important pathogen of small ruminants, but its antigenic proteins are not well known so far. In this study, we cloned the EF-Tu gene of *M. ovipneumoniae* and analyzed the molecular features of the gene and its coding protein for the first time. The gene was then expressed in *E.coli* and the antigenicity of the coding protein was evaluated as well. The EF-Tu gene of *M. ovipneumoniae* is 1209 bp in length, encodes 402 amino acids, and shares the highest DNA sequence identity of 87.5% and deduced amino acid sequence identity of 97.8% with those of *M. hyopneumoniae*, respectively. The recombinant EF-Tu protein can react with the polyclonal antiserum of *M. ovipneumoniae* and can induce humoral immune responses in mice, which indicated that the EF-Tu may be used as a candidate protein in developing the technologies to control the disease.

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INTRODUCTION

Mycoplasma ovipneumoniae, belongs to the Mycoplasmaceae, is considered to be a cause of non-progressive pneumonia of both sheep and goats (Niang *et al*., 1998; Besser *et al*., 2008). As well as causing disease in its own right, *M. ovipenumoniae* predisposes animals to invasion by more serious respiratory pathogens, such as *Mannheimia haemolytica*, *Pasteurlla* and *Parainfluenza 3 virus* (Dassanayake *et al*., 2010). Compared to other pathogenic mycoplasmas, less attention of research has been paid on *M. ovipneumoniae*, which could substantially impede understanding of the pathogenesis and developing technologies for control of *M. ovipneumoniae* infection.

Elongation factor (EF) is a major translational factor, which catalyzes the binding of aminoacyl-tRNA to the A-site of the ribosome (Andaleeb *et al*., 2011). It is not only a protein necessary for translation but also one of the most important multifunctional proteins. EF has been shown to be involved in various important cellular processes or diseases, including signal transduction, translational control, apoptosis, cytoskeletal organization, virus replication and oncogenic transformation (Zhou *et al*., 2007). Besides, EF-Tu can also provide beneficial effects against enteropathogen infection *in vitro* (Akhilesh *et al*., 2011). Elongation factor Tu (EF-Tu) of *M. hyopneumoniae* had already been identified as an antigenic protein (Pinto *et al*., 2007). Comparative analysis of known genomic sequences suggested that *M. ovipneumoniae* and *M. hyopneumoniae* share high homology, indicating that the two species of mycoplasma may exhibit similar mechanisms of active phenotypic switch and antigenic variation (Minion *et al*., 2004). The recent published un-annotated draft genomic sequences of *M. ovipneumniae* strain SC01 encouraged us to take the present study to evaluate if EF-Tu of *M. ovipneumoniae* is also an antigenic protein for potential use of disease control.

MATERIALS AND METHODS

Bacteria and sera: *M. ovipneumoniae* strain GH3-3 was an isolate from sheep in Gansu province, China (Xu *et al*., 2011). Polyclonal antiserum of *M. ovipneumoniae* was prepared as described before (Zhou *et al*., 2009).

Clone and synthesis of EF-Tu gene: EF-Tu gene sequence of *M. ovipneumoniae* was searched out from the draft genome of *M. ovipneumoniae* strain SC01 based on the EF-Tu gene sequence of the *M. hyopneumoniae* 232 strain (AE017332.1) using the MegAlign program of

Lasergene software version 7.1 (Madison, Wisconsin USA). The following primers, designed according to the EF-Tu gene sequence of SC01, were used to amplify EF-Tu gene of strain GH3-3: forward 5'-ATGGCAGTTGTTAAAACG-3'; reverse 5'-TTATTTAATAATTTCGGT-3'. The amplified product was purified and cloned into the pMD18-T vector (Takara Co., ltd., Dalian China) for sequencing. Then the EF-Tu gene sequence of *M. ovipneumoniae* strain GH3-3 was compared with that of strain SC01, and was optimized as follows to express in *E. coli:* (1) All TGA codons were changed into TGG; (2) *Xho* I and *Noc* I restriction enzymes cut sites were added at 3' and 5'-end, respectively; (3) Other optimization to improve the productiveness in *E.coli* BL21 (DE3) were made. The original and optimized EF-Tu gene sequences of strain GH3-3 were given in Fig. 1. The optimized EF-Tu gene was synthesized and cloned into the plasmid pUC57-simple in GenScript Co., Ltd. (Nanjing, China).

Molecular features of the EF-Tu gene and protein: The EF-Tu gene and protein sequences of 11 known Mycoplasma species collected from BLAST program (http://www.ncbi.nlm.nih.gov/blast) were used to compare with *M. ovipneumoniae* strain GH3-3 using MegAlign program. The primary structure of the EF-Tu protein, including physical and chemical property, and trans-membrane domain, was predicted using ExPASy software tools (http://www.expasy.ch/tools/#primary). The prediction of protein secondary structure, including α-helix, β-sheet, turn and coil, flexibility, hydrophilicity, antigenic index, and surface probability, was scored using Protean program of Lasergene software version 7.1. The signal peptide of the protein was predicted by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The potential antigen epitopes were predicted using DNAMAN software (Lynnon Biosoft, USA).

Expression, purification and Western blotting of recombinant EF-Tu: The synthesized EF-Tu gene was sub-cloned into plasmid pET32a (Invitrogen, USA) and was then transformed into *E.coli* BL21 (DE3) (TranGen Biotech Co., ltd., Beijing China) by using regular heat-shock transformation method. A single positive clone was picked into 10 ml of Luria-Bertani (LB) broth and cultured at 37°C. Isopropyl β-D-1-Thiogalactopyrano-side (IPTG) was added at a final concentration of 0.5 mM for preliminary induction to express when the OD_{600} of the culture reached 0.4. After an additional 8 h of cultivation, the culture broth was centrifuged at 1200x g for 1 min. The harvested cells were suspended in pH 7.2 Phosphate Buffered Saline (PBS) and three times volume of protein loading buffer (4×) (Takara Co., ltd., Dalian China) was then added and mixed. The cells were lysed by boiling for 10 min. After that the bacterial protein lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The expression conditions were optimized with different temperature of 28°C, 33°C, 37°C and different IPTG final concentration of 0.25 mM, 0.5 mM, 1.0 mM, respectively. The recombinant His-tagged EF-Tu protein was purified using a Ni–NTA column (Novagen, USA) and was then stored at -20°C until use. To test the immuno-reactivity of recombinant EF-Tu protein, Western blotting was performed using rabbit anti-*M. ovipneumoniae* polyclonal serum as primary antibody and goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (Zhong Shan Golden Bridge Bio Co., Ltd., Beijing China) as secondary antibody.

Immunization and sera collection: SPF BALB/c female mice (6-8 weeks old) (Lanzhou Institute of Biological Products, Lanzhou China) were randomly divided into five groups (five mice each group). Group 1, 2 and 3 were subcutaneously vaccinated with 40, 20, 10 μg of recombinant EF-Tu protein emulsified in Freund's complete adjuvant (Sigma, Saint Louis USA) and boosted using emulsion with Freund's incomplete adjuvant (Sigma, Saint Louis USA) at 15 and 30 days post first injection. Group 4 and 5, as control groups, received an equal volume of PBS and PBS+adjuvant, respectively. Blood samples were collected from the tail vein at 0, 15, 30 and 45 days post inoculation (dpi), respectively. The sera were separated and stored at -20°C until use.

Serum antibody detection: ELISA was employed to detect antigen-specific antibody in the sera of mice. Briefly, 96-well microtiter plates were coated overnight at 4°C with 10 μg/ml recombinant EF-Tu protein. After washed with PBST (PBS with 0.05% Tween-20) for 4 times, 50 μl of serum diluted at 1:10 in PBST was added and incubated at 37°C for 1 h, in triplicate. After wash, HRP-conjugated goat anti-mouse antibody (Beijing Solarbio Science & Technology Co, Ltd, Beijing, China) diluted in PBST at 1:800, was added and incubated at 37°C for 1 h. The reactions were developed with 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) after the final washes steps. OD_{450} were measured in an ELISA Reader (Bio-rad, Philadelphia USA) 10 min later.

Antibody titers were analyzed using One-way ANOVA algorithm of software SPSS version 19 (SPSS Co., Ltd., USA). The differences of data were considered significant if $P \leq 0.05$.

RESULTS

Characteristics of EF-Tu gene and its coding protein: The EF-Tu gene of *M. ovipneumoniae* strain GH3-3 was successfully amplified. Its sequence was consistent with that of strain SC01. The full-length of EF-Tu gene was 1209 bp with a predicted molecular mass of 1098.5 kDa and with a (G+C) % content of 39.62%. Comparative analysis of EF-Tu DNA and amino acid sequence revealed that GH3-3 had 28.7 to 87.5% identity of DNA sequence and 6.8 to 97.8% identity of amino acid sequence with those of 11 other mycoplasmas, respectively. EF-Tu of *M. hyopneumoniae* was closest to that of GH3-3(87.5% of DNA and 97.8% of protein sequence identity). EF-Tu protein contains 402 amino acids with a predicted molecular weight of 43.863 kDa. Theoretical pI was 5.70, total number of negatively (Asp+Glu) or positively (Arg+Lys) charged residues is 61 or 52. Formula was $C_{1944}H_{3131}N_{531}O_{596}S_{12}$, total number of atoms was 6214. The estimated half-life was more than 10 hours (*E.coli*, *in vivo*). The instability index (II) was

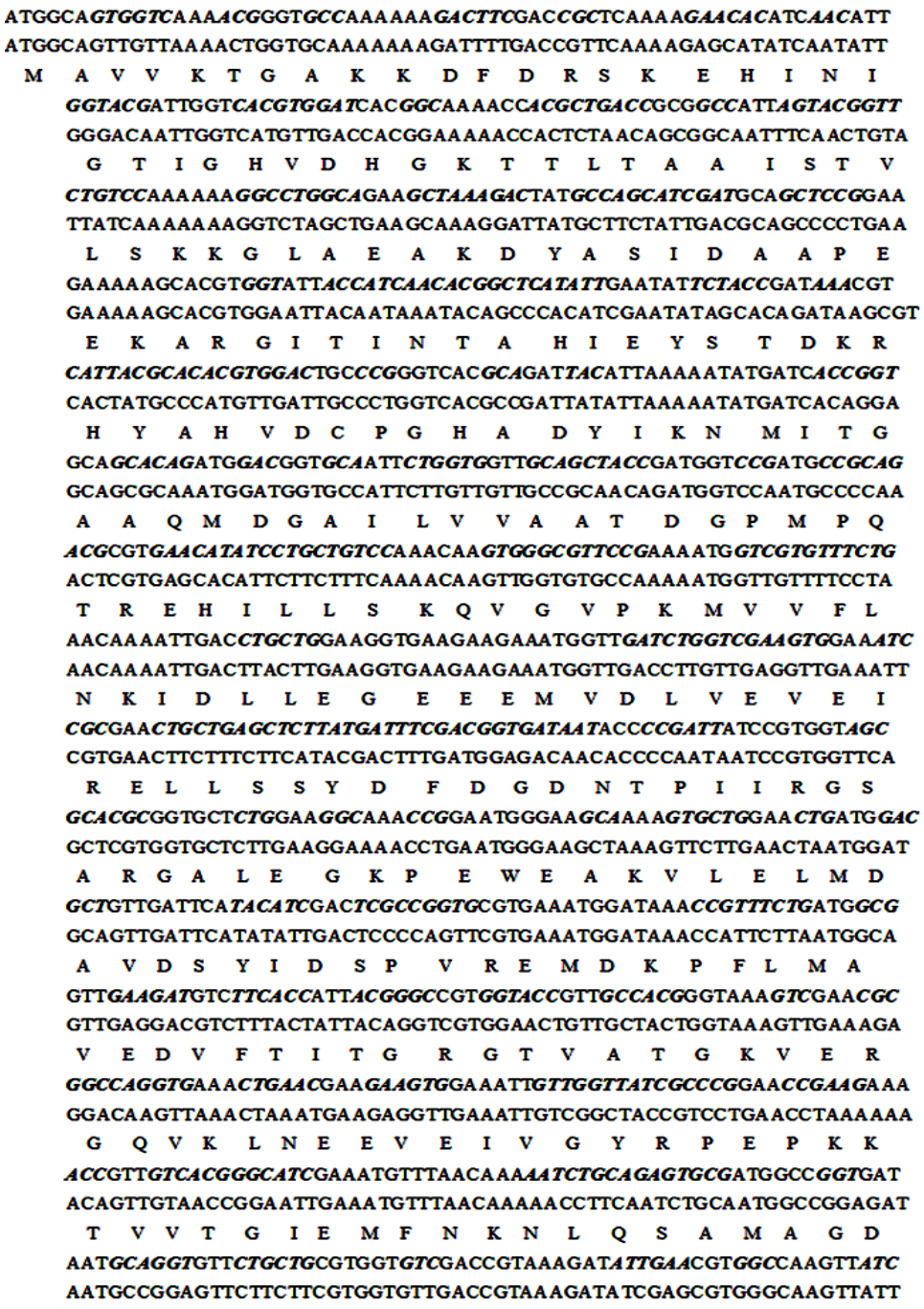

Fig. 1: Original and optimized DNA sequence of *M. ovipneumoniae* EF-Tu and its amino acid sequence. The bold and italic letters in the first line represent optimized nucleotides. The second line represents original sequences. The third line represents the amino acid sequence.

computed to be 32.61, which classified the protein as stable. Secondary structure analysis indicated the protein contained a large number of α-helix and turns, but no trans-membrane region and signal peptide. Antigen epitopes prediction showed that the protein include 16 antigenic epitopes, which would provide a theoretic clue for development of epitope-based vaccines against *M. ovipneumoniae* infection.

Expression, purification and Western-blot analysis of EF-Tu: The recombinant plasmid pET32a-*EF-Tu* was validated by digestion with restriction enzymes of *Nco* I and *Xho* I and sequencing of the insert. The recombinant EF-Tu of approximately 44 kDa was well expressed at 37°C for 8 h with a concentration of 0.5 Mm IPTG. A high degree of purity of the recombinant protein was obtained after purification using Ni–NTA column. Western-blot suggested that the recombinant EF-Tu protein can react specifically with the serum against *M. ovipneumoniae* (Fig. 2).

Serum antibody detection: The mean antibody titers to EF-Tu protein detected in mice sera from all groups by ELISA were shown in Fig. 3. In general, antibody levels

of mice from 3 immunized groups post vaccination were all significantly higher than that of pre-immune (P<0.05), that of PBS control group (P<0.05) and that of PBS+adjuvant control group (P<0.05). After the first immunization, all immunized mice produced considerable antibody responses (mean antibody titers) when compared to both controls (P<0.05), but there were no significant differences among the three immunized groups (P>0.05). The antibody titers of three immunized groups after the second immunization all appeared slight increase while there were no significant differences compared with first immunization (P>0.05), and no significant differences among immunized groups was observed. After the third immunization, antibody level of three immunized groups did not rise significantly (P>0.05, compared with the first and second immunization) and no significant differences among immunized groups were observed, but maintained at a steady level of antibodies which suggested that immunized mice with recombinant EF-Tu protein had arrived at a considerable and best antibody level after the second immunization. It was noted that the antibodies to recombinant EF-Tu were produced in mice from group of PBS+adjuvant after first injection, although there was no significant difference between the two control groups (P>0.05).

DISCUSSION

EF-Tu is one of the most important multifunctional proteins in organisms. A number of studies have demonstrated that EF-Tu is one of the most abundant bacterial proteins and conserved bacterial proteins and is a major component of the bacterial membrane cytoskeleton (Mayer, 2003; Jonak, 2007). EF-Tu constitutes approximately 10% of total proteins in all bacterial investigated such as *M. pneumonia* (Regula *et al*., 2000). Recent evidences demonstrate that EF-Tu may play a previously under-appreciated role as a bacterial virulence factor and have a significant effect to facilitate invasion of host cells of *M. pneumonia* and *Pseudomonas aeruginosa* (Dallo *et al*., 2002; Kunert *et al*., 2007; Balasubramanian *et al*., 2008; Barel *et al*., 2008). Furthermore, some researches have identified EF-Tu as an immunodominant protein through immunoproteomic-based approaches for antigen discovery against other intracellular bacterial pathogens and mycoplasma (Bunk *et al*., 2008; Gupta *et al*., 2009; Nieves *et al*., 2010; Xu *et al*., 2012). EF-Tu of *M. mycoides subsp. mycoides* small-colony type (MmmSC) and *M. hyopneumoniae* strain 7448 had also been identified as a potential antigen (Alonso *et al*., 2002; Pinto *et al*., 2007). Taken together, all these achievement could provide support for our observations of immunogenic EF-Tu in the membrane of *M. ovipneumoniae*. In the present study, Western-blot showed that the recombinant EF-Tu of GH3-3 can be recognized by polyclonal serum of *M. ovipneumoniae*, it was proved to have a strong reactivity. By immunizing to mice the immunogenicity of EF-Tu was further confirmed. The results suggested that the EF-Tu is an antigenic protein of *M. ovipneumoniae*. However, the antibody responses among three groups of mice immunized with different doses of proteins have no significant differences, which indicated that the

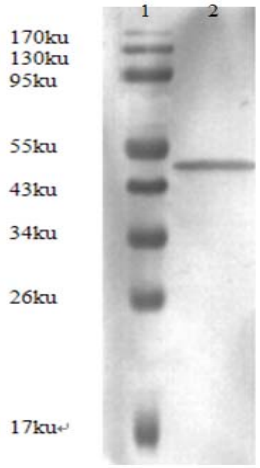

Fig. 2: Western blotting of the recombinant EF-Tu protein using antiserum of *M. ovipneumoniae*. 1, protein marker; 2, purified recombinant EF-Tu.

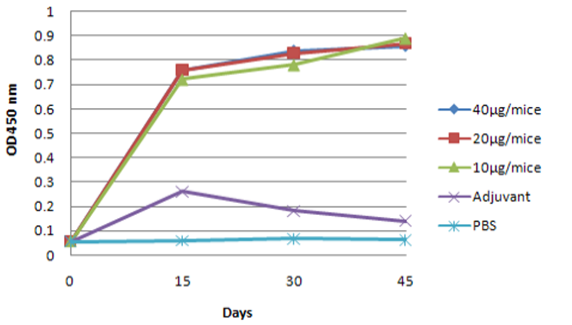

Fig. 3: Detection of antibody titers in srea from mice immunized with EF-Tu and PBS. Data points mean the average OD_{450} value.

concentration of EF-Tu protein within the given range has less effect to antibody titers specific for EF-Tu antigen. On the other hand, although the antibody level of immunized mice did not increase significantly after the second and third immunization, a high level of antibody was maintained during the experiment when compared with the control group. The results suggested that EF-Tu has the ability to stimulate antibody production in mice.

The PBS+adjuvant control group raises an antibody titer after first immunization, perhaps because Freund's complete adjuvant contains *Mycobacterium tuberculosis* cell wall components. Based on the assumption, we conducted a homology comparison of EF-Tu amino acid sequence between *M. tuberculosis* and *M. ovipneumoniae*. The result showed that they share an identity of 67.5%, which may be a possible cause of producing antibody against EF-Tu of *M. ovipneumoniae*. It indicated that EF-Tu cannot be a candidate for developing specific diagnostic methods depended on antibody detection. The antibody interference from Freund's complete adjuvant should be considered in the similar experimental design.

In some animal diseases caused by other mycoplasmas, immune protection has not been achieved when antibody responses can be obtained (Shahzad *et al*., 2010). So the potential of EF-Tu of *M. ovipneumoniae* as a candidate vaccine protein should be further investigated, especially on cell-mediated immune responses of host.

Conclusion: EF-Tu of *M. ovipneumoniae* is characterized and identified as an antigen. It can be further studied for a purpose of developing the molecular vaccine for *M. ovipneumoniae* infection.

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