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Mycoplasma synoviae elongation factor thermo stable is an adhesion-associated protein that enters cells by endocytosis and stimulates DF-1 cell proliferation

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Abstract

Mycoplasma synoviae is an important avian pathogen that causes respiratory infections and arthritis symptoms in chickens and turkeys, resulting in significant economic damage to the poultry farming industry worldwide. Cell adhesion is a vital stage of *Mycoplasma* infection, and the proteins associated with this process play an important role in its pathogenesis. Elongation factor thermo stable (EF-Ts) is an important factor in prokaryotic biosynthesis that serves as a guanosine exchange factor for elongation factor thermo unstable (EF-Tu). To date, little is known about the role of EF-Ts in *Mycoplasma* infection. In this study, we identified EF-Ts as an immunogenic protein in *M. synoviae* through liquid chromatography with tandem mass spectrometry (LC–MS/MS) screening. We constructed an *E. coli* recombinant expression vector and prepared a highly efficient rabbit antiserum. Immunoblot analysis and suspension immunofluorescence revealed that the EF-Ts is located in both the cell membrane and cytoplasm. The prepared rabbit EF-Ts antiserum exhibited complement-dependent *Mycoplasma*-killing activity and inhibited the adhesion of rEF-Ts and *M. synoviae* to DF-1 cells. An *in-vitro* binding assay showed that EF-Ts could bind to fibronectin (Fn) and chicken plasminogen (cPlg) in a dose-dependent manner. In addition, EF-Ts could internalize into cells through lipid rafts and clathrin-dependent endocytosis and induce DF-1 cell proliferation. In conclusion, our studies demonstrated that MS EF-Ts is a potentially immunogenic, novel adhesion protein that acts as a critical virulence factor in *M. synoviae* adhesion to host cells during infection. These studies further deepen our understanding of the pathogenic mechanism of *M. synoviae*.

Keywords *M. synoviae*, Elongation factor thermo stable, Membrane proteins, Adhesion, Endocytosis

Introduction

Mycoplasma synoviae (*M. synoviae,* MS*)* infects chickens and turkeys, causing respiratory, arthritis, and reproductive tract-related diseases that reduce carcass quality and egg production in sick poultry [1]. *Mycoplasma* infections weaken the immune system and can aggravate respiratory disease or systemic symptoms during coinfection with Newcastle disease virus, avian influenza virus, infectious bronchitis virus, and infectious bursal virus [2, 3]. *M. synoviae* infection was first reported in the United States in 1954 and has since been detected in Canada, the United Kingdom, Norway, Germany, and France [4]. In the Netherlands, a decline in the prevalence of *Mycoplasma gallisepticum* was observed, while the seroprevalence

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of *M. synoviae* in laying flocks exceeded 70%. Additionally, evidence indicated that *M. synoviae* was becoming a more significant source of infection in poultry, superseding *Mycoplasma gallisepticum* [5]. Although *M. synoviae* rarely causes direct mortality, it results in significant economic damage to the poultry farming industry [6]. For example, in poultry farms throughout Quebec, Canada, more than 50% of the flocks sampled were positive for *M. synoviae* [7]. In addition, in a serological survey of eastern Spain, *M. synoviae* seropositivity in laying hens and broilers was 95% and 74%, with infection prevalence rates of 95% and 35%, respectively [8]. In China, overall *M. synoviae* positivity rates reached 50% from 2010–2015, with the affected area gradually extending inland from more humid regions, revealing a nationwide epidemic [9, 10]. Therefore, *M. synoviae* infection has become a serious problem for the poultry industry worldwide. Research into the pathogenic mechanisms and virulence factors of *M. synoviae* will contribute to the development of diagnostic reagents, vaccines, and therapeutic drugs.

Adhesion is the first step in colonization during pathogen infection; therefore, bacterial adhesins can be considered promising vaccine candidates [11]. As *Mycoplasma* lack cell walls, membrane surface lipoproteins play an important role in the adhesion and invasion of host cells and are critical for activating the host immune response; thus, these proteins are potential vaccine candidates [11, 12]. Despite the screening of several *M. synoviae* immunogenic adhesion-associated proteins in recent years, including α-Eno [13], NADH [14], DLD [15], PDHA [16], PDHB [16], P35 [17], P78 [18], VlhA [19], and EF-Tu [19], significant challenges persist in designing an effective *M. synoviae* vaccine. Membrane proteins are key *Mycoplasma* proteins and are integral to various microbial activities. Recent studies have demonstrated that multiepitope subunit vaccines constructed with immunogenic membrane proteins significantly enhance protection against *M. synoviae* [20, 21]. Therefore, the identification and screening of *M. synoviae* immunogenic proteins is beneficial for vaccine development and infection prevention.

Elongation factor thermo stable (EF-Ts) is a prokaryotic translation elongation factor vital to prokaryotic biosynthesis processes [22]. EF-Ts is potentially a multifunctional protein that significantly impacts bacterial pathogenicity and drug resistance and has been identified as immunogenic in numerous pathogens, including *Pasteurella multocida*, *Helicobacter pylori* Antigens, and *Vibrio parahaemolyticus* [23–25]. EF-Ts have been discussed in numerous studies on the identification of immunogenic proteins in various *Mycoplasma* species and is a known secreted protein in *Mycoplasma bovis* and *Mycoplasma mycoides subsp.* [26]. Although the immunogenicity and ability of EF-Ts to induce host immune responses have been explored in some pathogens, the function and role of EF-Ts in the adhesion and invasion of *M. synoviae* are unknown.

In this study, we investigated the pathogenesis and conditions of *M. synoviae* infection by examining the role played by *M. synoviae* proteins during *Mycoplasma* adhesion, invasion, and internalization. We identified the immunogenicity of EF-Ts in *M. synoviae* by liquid chromatography with tandem mass spectrometry (LC–MS/MS) (supplemental file 2) and illustrated the novel function of EF-Ts in the adhesion and invasion of host cells, indicating that EF-Ts is a promising target for the development of therapies and vaccines.

Materials and methods

Bacterial strains, cell lines, plasmids, and cultivation

M. synoviae strain WVU1853 was obtained from the China Veterinary Conservation Centre (CVCC, Beijing, China) and cultured in *Mycoplasma* medium base (Haibo, Qingdao, China) supplemented with 0.01% NAD (Roche, Shanghai, China), 1% L-cysteine (Solarbio, Beijing, China), 80,000 units of penicillin, and 10% swine serum (Minhai, Lanzhou, China) at 37 °C in 5% atmospheric CO_2. DF-1, a continuous cell line of chicken embryo fibroblasts, was obtained from Procell (Wuhan, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA). The cultured DF-1 was then supplemented with 10% fetal bovine serum (FBS; Biological Industries, Israel) at 37 °C in 5% atmospheric CO_2. *Escherichia coli (E. coli)* strains DH5α and BL21 (DE3) were cultured in Luria–Bertani (LB) broth or on solid media with 1.5% agarose, both of which were supplemented with 30 μg/mL kanamycin. pET-28a (+) expression vectors were obtained from Novagen (Germany).

Cloning, expression, and purification of EF-Ts

The *M. synoviae* strain WVU1853 was collected at the logarithmic growth stage, and the whole genome was extracted with a bacterial genomic DNA extraction kit (Tiangen, Beijing, China). According to the GenBank database (GenBank accession no. CP011096.1, *M. synoviae* ATCC 25204), there are two tryptophan (TGA) codons in the CDS of *M. synoviae* EF-Ts that play a role in terminating expression in *E. coli*. Therefore, in this study, we designed three pairs of specific primers (Table 1), amplified the full-length sequence of *M. synoviae* EF-Ts by overlapping PCR to mutate the TGA point to TGG, and added the *Bam*HI and *Xho*I restriction enzyme cleavage sites at both ends. The optimized *M. synoviae* EF-Ts gene was cloned and inserted into the pET-28a (+) expression vector and transfected into

Table 1 Primers used in this study

Primer	Sequence (5′-3′)	Localization
EF-Ts-F1	GGA*GGATCC*[a]ATGTCACAAAACAAACTAGAAT	1–132
EF-Ts-R1	GCG*CTCGAG*[a]TTATTTTGTCATTGAAGCAACT	
EF-Ts-F2	GCAATTAAATG**G**[b]TTAAAAGAAAAC	109–696
EF-Ts-R2	GTTTTCTTTTAA**C**[b]CATTTAATTGC	
EF-Ts-F3	TCAGGATG**G**[b]CTTGAC	682–879
EF-Ts-R3	GTCAAG**C**[b]CATCCTGA	

[a] Limitations of the endonuclease *Bam*HI and *Xho*I checkpoints GGATCC and CTC GAG are shown in underlined italics

[b] Nucleotide point mutation site

E. coli BL21 (DE3) cells by heat excitation. The expression of recombinant *M. synoviae* EF-Ts was induced by isopropyl-β-D-thiogalactopyranoside (IPTG; Solarbio, Beijing, China) at a final concentration of 1 mM at 16 °C overnight, and the fusion proteins were obtained by Ni–NTA His-Tag purification Agarose (HY-K0210, MCE). The purified proteins were assayed for protein quantity using the BCA Protein Assay Kit (Beyotime, Shanghai, China), and the recombinant proteins were identified by 10% SDS–PAGE with Coomassie blue staining.

EF-TS polyclonal antiserum preparation and immunogenicity characterization

Polyclonal antibodies against *M. synoviae* EF-Ts protein were obtained by immunization of one-month-old New Zealand White rabbits with purified recombinant EF-Ts protein. Purified rEF-Ts proteins were emulsified with complete (first immunization; 800 μg of protein) or incomplete (second to fourth immunizations; 400 μg of protein) Fuchs adjuvant (1:1, v/v). The second immunization was performed two weeks after the first immunization; subsequent immunizations were given at one-week intervals for four immunizations total. The serum was collected 3 days after the fourth immunization and analyzed by enzyme-linked immunosorbent assay (ELISA) to determine the antibody titer. Briefly, 96-well plates were coated with 5 μg/mL rEF-Ts protein for 2 h at 37 °C. After blocking with 5% skim milk, rEF-Ts antisera and preimmune serum were added to the wells in serial dilutions and incubated for 1 h at 37 °C, followed by incubation with a 1:5,000 dilution of goat anti-rabbit IgG-HRP. After termination of 3,3′,5,5′-tetramethylbenzidine (TMB) color development with H_2SO_4, the optical density (OD) was read at 450 nm, and the maximum dilution titer satisfying the criterion (OD positive/OD negative > 2.1) was recorded as the antibody titer. The specificity of the resulting polyclonal antibodies was verified using western blotting. In accordance with the aforementioned methodology, *M. synoviae* particles were combined with an adjuvant, and rabbits were immunized in order to obtain *M. synoviae* 1853 antiserum.

Analysis of complement-dependent killing of *M. synoviae*

Complement-dependent killing of *M. synoviae* was assessed as previously described with slight modifications [15]. Briefly, 2 ml of *M.synoviae* in the logarithmic growth phase in broth was collected, and the precipitate was obtained by centrifugation at 8,000 r/min for 10 min and resuspended in 1 ml of sterile PBS. The *M. synoviae*-immunized rabbit sera, rEF-Ts-immunized rabbit sera, and non-immunized rabbit sera were inactivated at 56 °C for 30 min. Sixty microliters of each inactivated serum sample was mixed with 180 μl of resuspended bacterial solution, then incubated at 37 °C for 30 min, supplemented with 60 μl of complement (freeze-dried complement, BerseeTech, Beijing, China; complement activity: ≥ 100 U/ branch), and mixed well. After 1 h of incubation, a tenfold dilution was prepared, and three gradients, namely, 10^{-3}, 10^{-4}, and 10^{-5}, were used to coat 60 mm solid media plates (100 μl/well). Each gradient was used for three parallel replicates. Colonies were counted after incubation at 37 °C in 5% atmospheric CO_2 for 3–5 days. Anti-*M. synoviae* serum, anti-rEF-Ts serum, nonimmunized serum, and blank control (60 μl of PBS instead of serum) were used for the experiment, and the experiment was repeated 3 times. The bactericidal rate was calculated according to the following formula: [(CFU from nonimmunized serum treatment—CFU from antiserum treatment)/CFU from nonimmunized serum treatment) × 100%].

EF-Ts localization analysis of *M. synoviae*

One hundred milliliters of *M. synoviae* cultured to the logarithmic growth stage was collected and resuspended in 1 ml of 1 × PBS. PMSF solution at a final concentration of 1 mmol/L and Triton X-114 at a final concentration of 1% were added to the *Mycoplasma* suspension. After incubation on a shaking table at 4 °C for 2 h (with 100 μl of the suspension collected as whole bacterial protein), the supernatant was collected by centrifugation and incubated in a water bath at 37 °C for 10 min, followed by centrifugation at 12,000 r/min at 4 °C for 10 min. The upper aqueous and lower oil phases were separated, formaldehyde/chloroform (4/1) was added to the precipitated proteins, the mixture was centrifuged at 10,000 r/min for 10 min, and an equal volume of 8 M urea was added to dissolve the proteins. The mixture was then stored at −20 °C after quantification using the BCA Protein Assay Kit. The distribution of the *M. synoviae* EF-Ts protein was determined using western blotting and ELISA.

Suspension immunofluorescence assay

The presence or absence of EF-Ts in the cell membrane was determined using the suspension immunofluorescence technique as previously described, with modifications [15]. Two milliliters of logarithmic growth phase *M. synoviae* strain WVU1853 was collected by centrifugation and fixed with 4% paraformaldehyde for 15 min, after which it was subsequently blocked with 5% skim milk solution at room temperature for 1 h. After incubation with rabbit anti-EF-Ts or preimmune serum (1:200) for 1.5 h at 37 °C, the cells were incubated with goat anti-rabbit IgG H&L/FITC secondary antibody (bs-0295G-FITC, Bioss, China) for 1 h and washed with 1×PBST (made by adding a final concentration of 0.1% Tween-20 to 1×PBS). The *M. synoviae* were spread onto glass slides, and the samples were examined using a Zeiss Observer A1 inverted fluorescence microscope (Zeiss, Oberkochen, Germany).

Analysis of EF-Ts binding to DF-1 cells

Membrane proteins were extracted from DF-1 cells using a Membrane Protein Extraction Kit (Solarbio, Beijing, China). ELISA was performed as previously described [14]: 500 ng of cell membrane proteins were encapsulated in each well of the ELISA plate, which was blocked using 5% skim milk. rEF-Ts and anti-rEF-Ts serum pretreated with rEF-Ts were added to the wells. Rabbit anti-rEF-Ts serum was used as the primary antibody, and goat anti-rabbit IgG H&L/HRP (bs-0295G-HRP) was used as the secondary antibody.

Cell immunofluorescence was used to determine protein adhesion as previously described [14]. DF-1 cells were incubated overnight in 12-well plates, the original medium was removed, and the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and blocked with 5% BSA. The plates were incubated with 10 μg of rEF-Ts or BSA at 37 °C for 2 h. Anti-EF-T serum was used as the primary antibody, goat anti-rabbit IgG H&L/FITC was used as the secondary antibody, DiI was used to mark the cell membranes, and DIPA was used to mark the nucleus. Following washing, the cells were observed using a Leica Deltavision Ultra Acquire Ultra microscope.

Adhesion inhibition analysis of anti-EF-Ts serum

The adhesion inhibition assay was performed as previously described with slight modifications. The rEF-Ts and *M. synoviae* were pretreated with rabbit anti-rEF-Ts serum or nonimmunized rabbit serum (1:50) for 1 h at 37 °C and added to the cells. After 2 h of incubation, washing, and fixation, the cells were stained with antibodies and observed under a Leica Deltavision Ultra Acquire Ultra microscope.

The ability of rabbit anti-rEF-Ts serum to inhibit *M. synoviae* adhesion was examined by colony counting assay as previously described with some modifications [27]. Briefly, *M. synoviae* was pretreated with rabbit anti-rEF-Ts serum, rabbit anti-*M. synoviae* serum, or nonimmunized rabbit serum for 1 h at 37 °C. DF-1 cells from each group were incubated with *M. synoviae* treated with antiserum at a multiplicity of infection (MOI) of 500 for 2 h at 37 °C. Nonadherent *Mycoplasma* were then removed by washing three times with sterile PBS. The cells were lysed in 1 ml of pure water and resuspended in serial tenfold dilutions. One hundred microliters of each dilution were spread on a *Mycoplasma* agar plate for colony counting. Three independent experiments were performed in triplicate.

EF-Ts binding to chicken Plg (cPlg) and human Fn (hFn)

The binding activities of EF-Ts to cPlg and hFn were determined by ELISA and dot blot analysis [28]. For the dot blot analysis, cPlg and hFn (5.0–0.3125 μg) were added dropwise in serial twofold dilutions to nitrocellulose filter membranes (NCs), with BSA as the negative control. The NC membranes were dried at 37 °C for 2 h, after which the NC membranes were blocked with 5% skim milk for 1 h at room temperature. The NC membranes were subsequently incubated with 10 μg of rEF-Ts overnight at 4 °C. The membranes were washed with PBST, rabbit anti-rEF-T serum was used as the primary antibody, and goat anti-rabbit IgG H&L/HRP was used as the secondary antibody. Finally, membrane signals were detected using enhanced chemiluminescence (ECL) as described above.

For the ELISA, 96-well enzyme-labeled plates were coated with cPlg or hFn (1 μg/well) at 4 °C and incubated overnight. After blocking with 5% skim milk, serially diluted EF-Ts or BSA were bound to the plates at 37 °C for 2 h. The samples were washed with PBST, and the absorbance was measured at 450 nm using rabbit anti-rEF-Ts serum as the primary antibody and goat anti-rabbit IgG H&L/HRP antibody as the secondary.

rEF-Ts endocytosis pathway studies

For the colocalization studies, 20 μg of rEF-T protein was incubated with DF-1 cells at 37 °C for 2 h, after which anti-rEF-T serum, Cav-1 monoclonal antibody (66,067–1-Ig, Proteintech), and CLTC monoclonal antibody (66,487–1-Ig, Proteintech) were used as primary antibodies, and goat anti-mouse IgG H&L/Cy3 antibody (bs-0296G-Cy3, Bioss, China) and goat anti-rabbit IgG H&L/FITC antibody were used as secondary antibodies. The co-localization of rEF-Ts with caveolin and clathrin was examined using laser confocal microscopy. The endocytosis inhibition assay was modified as previously

described to detect the endocytosis pathway of rEF-Ts using two specific inhibitors [29, 30]. Pitstop-2 (HY-115604, MCE) is an inhibitor of clathrin protein-mediated endocytosis, and simvastatin (HY-17502, MCE) is an inhibitor of caveolin protein pathway-mediated endocytosis. DF-1 cells were treated with 25 μM simvastatin or 20 μM Pitstop-2 for 30 min and incubated with rEF-T proteins for 2 h at 37 °C and 5% CO2. The cytoskeleton was labeled with Actin-Tracker Red-555 (Beyotime, Shanghai, China) and analyzed using a Leica Deltavision Ultra Acquire Ultra microscope.

Cell Counting Kit-8 (CCK-8) analysis

DF-1 cell proliferation was assayed using CCK-8 (Beyotime, Shanghai, China), and the results were analyzed according to the manufacturer's instructions. DF-1 cells (4×10^3) were inoculated into 96-well plates and cultured overnight. The medium was changed to DMEM supplemented with 2% FBS, different concentrations of rEF-Ts protein were added to the wells, and the cells were incubated for 24 h, 48 h, or 72 h. Then, CCK-8 solution (10 μl) was added to each well, the plates were incubated for 2 h, and the OD was detected at 450 nm.

Treatment of laboratory animals

The New Zealand white rabbits utilized in the experiment were humanely euthanized through anesthetic bloodletting. An intravenous injection of sodium pentobarbital (100–200 mg/kg) was first administered to induce deep anesthesia, after which an incision was made in the aorta, resulting in bleeding that led to cardiac arrest.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.0. Student's t-test or ANOVA was used for multiple comparisons. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$. ns, no significant difference ($p\geq0.05$).

Results

Immunogenicity analysis of rEF-Ts

Excellent immunogenicity and reactogenicity are key in screening possible *M. synoviae* candidate antigens for vaccine development. The rEF-Ts protein with a His-tag was successfully expressed in *E. coli* BL21 (DE3), and the protein was purified and separated by 10% SDS–PAGE. However, a stray band at 42KDa was found to be present regardless of elution, corresponding to the *E. coli* EF-Tu protein. A single band was obtained by denaturing rEF-Ts according to a previously published method [31]. (Fig. 1A). Purified rEF-Ts could bind specifically to the rEF-Ts rabbit antiserum but did not react with non-immunized rabbit serum (Fig. 1B). Thus, rEF-Ts can stimulate host-specific antibody production. To confirm whether EF-Ts can be used as a broad-spectrum vaccine against *M. synoviae*, we performed western blot analyses on eight clinical isolates with rabbit antiserum rEF-Ts. EF-Ts (37 kDa) was detected in all the isolates, indicating that EF-Ts is highly conserved in different *M. synoviae* isolates and can be stably expressed (Fig. 1C). These results suggest that EF-Ts is highly immunogenic and a candidate vaccine for *M.synoviae*.

Surface localization of *M. synoviae* EF-Ts

To determine the distribution of EF-Ts in *M. synoviae*, we extracted *M. synoviae* cell membrane and cytoplasmic proteins and measured them in anti-rEF-Ts serum (Fig. 2A and B). The presence of EF-Ts in the cell membrane was further demonstrated by suspension immunofluorescence detection (Fig. 2C).

Complement-dependent *Mycoplasma* killing assay

The bactericidal rate was calculated by colony counting in the complement-killing *Mycoplasma* test. The complement-mediated *Mycoplasma* killing rate activated by anti-*M. synoviae* serum was 70.5%, whereas that activated by the rEF-Ts multi-antiserum was 33.75% (Table 2). This finding indicates that rEF-Ts antiserum activates complement and has *Mycoplasma*-killing activity.

Adhesion of rEF-Ts to DF-1 cells

The interaction between rEF-Ts and DF-1 cells was analyzed by immunofluorescence and ELISA. Immunofluorescence analysis (Fig. 3C and D) revealed that rEF-Ts could adhere to the surface of DF-1 cells and that this effect could be inhibited by incubation with the rabbit antiserum rEF-Ts, as shown. The binding of rEF-Ts to cell membrane proteins was detected by ELISA to quantitatively analyze adhesion. Unlike the controls, 0.8–100 μg/mL rEF-Ts bound to DF-1 cell membrane proteins in a dose-dependent manner (Fig. 3A). This binding was specifically inhibited by anti-EF-Ts serum in a concentration-dependent manner (Fig. 3B). These results suggest that EF-Ts on the *Mycoplasma* surface directly bind to the DF-1 cell membrane, revealing EF-Ts as a novel *Mycoplasma* adhesion-associated protein.

Inhibition of *M. synovial* adhesion by rEF-Ts

The inhibition of *M. synoviae* adhesion to host cells by the rEF-Ts antiserum was determined by immunofluorescence (Fig. 4) and CFU counting (Table 2). In contrast to that observed with the treatment with nonimmunized rabbit serum, *M. synoviae* adherence to DF-1 cells was significantly reduced after incubation with the rEF-Ts antiserum. *Mycoplasma* adherence to DF-1 cells was significantly reduced after incubation with the rEF-T

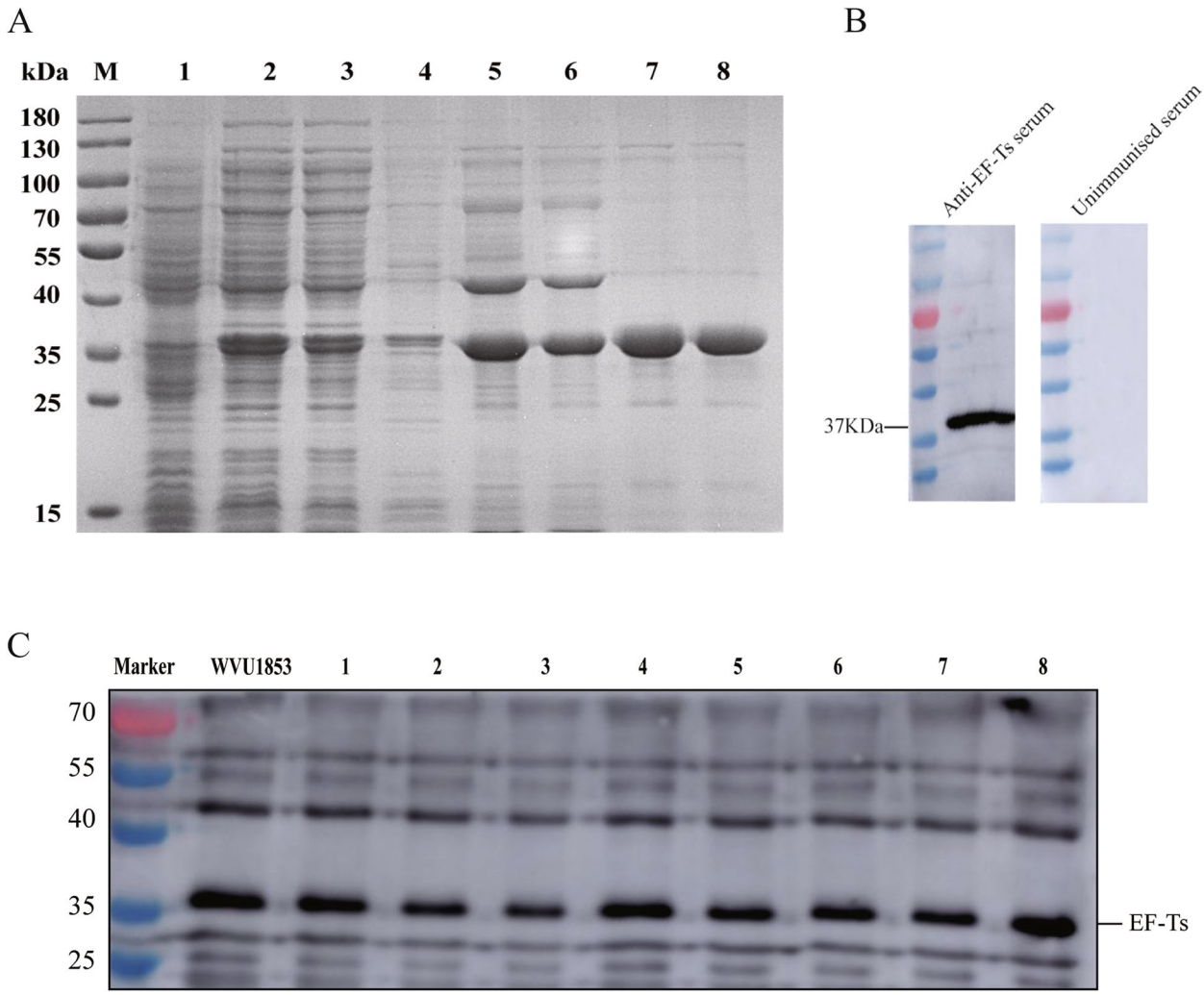

Fig. 1 Immunogenic analysis of EF-Ts in *M. synoviae*. **A** SDS–PAGE analysis of recombinant EF-Ts proteins. Protein molecular weight marker (M). Lysates of *E. coli* BL21 (DE3) containing pET-28a (+) empty vector (Lan1), whole-cell lysates of recombinant bacteria (Lan2), whole bacterial lysate supernatant (Lan3), whole bacterial lysate precipitation (Lan4), purified rEF-Ts protein not treated with 8 M urea (Lan5-6), and purified rEF-Ts protein treated with 8 M urea (Lan7-8) were obtained. **B** Rabbit anti-EF-Ts serum specificity analysis. The rEF-Ts was transferred to the NC membrane, and western blotting was performed with anti-EF-Ts serum (1:2 000) and nonimmunized serum (1:2 000). **C** The immunogenicity of EF-Ts in different *M. synoviae* strains. Whole bacterial proteins from *M. synoviae* strain WVU1853 and eight other wild-type isolates were transferred to NC membranes, incubated with a 1:2,000 dilution of anti-EF-Ts serum, and then detected with a 1:5,000 dilution of HRP-conjugated goat anti-rabbit antibody

antiserum, as shown in Fig. 4 and Table 3. These results further demonstrate that EF-Ts play an important role in *Mycoplasma* adhesion to host cells.

The ability of rEF-Ts to bind cPlg and hFn

The ability of rEF-Ts to bind to plasminogen and fibronectin was verified by dot blotting and ELISA, with BSA serving as a negative control, to explore the substrates for the adhesion of *M. synoviae* EF-Ts to the host cells. Binding assays showed that rEF-Ts could bind to plasminogen and fibronectin in a dose-dependent manner, and no binding of BSA was observed (Fig. 5).

rEF-Ts internalization pathway in DF-1 cells

The pathway involved in the internalization of DF-1 by rEF-Ts was investigated using a Leica Deltavision Ultra Acquire Ultra microscope. After the rEF-Ts were coincubated with DF-1, obvious fluorescence signals colocalizing with caveolin and clathrin were observed in the cells (Fig. 6). After treatment with the corresponding inhibitors, the fluorescence signal of rEF-Ts in the cells significantly decreased, indicating that the internalization of rEF-Ts by DF-1 cells is mediated by clathrin and caveolin (Fig. 7).

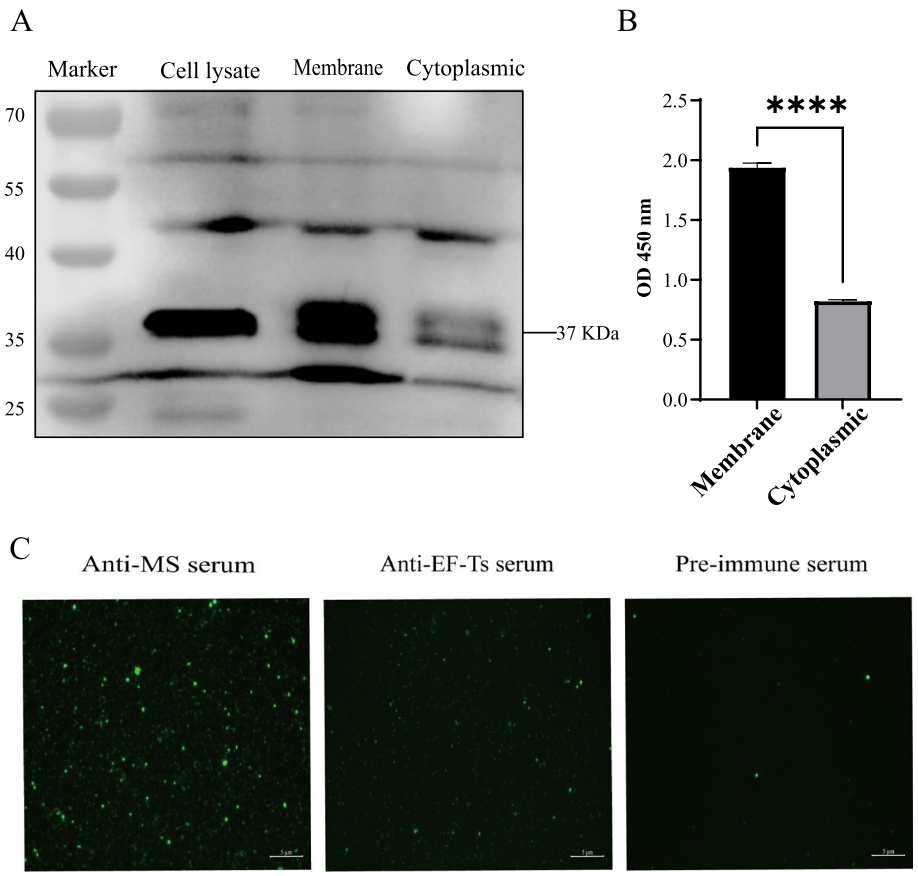

Fig. 2 Surface localization of EF-Ts in *M. synoviae*. **A** The distribution of EF-Ts in *M. synoviae* was detected by immunoblotting; 180-kDa protein marker (M). Western blotting of the total protein (Lan1), cell membrane fraction (Lan2), and cytoplasmic fraction (Lan3) of *M. synoviae* was performed using anti-EF-Ts serum. **B** Detection of EF-Ts in *M. synoviae* by ELISA. Equal concentrations of cell membrane and cytoplasmic proteins were encapsulated in enzyme-labeled plates and incubated with anti-EF-Ts serum for 1 h, followed by incubation with an HRP-coupled goat-anti-rabbit secondary antibody and color development with tetramethylbenzidine. The absorbance values at 450 nm were read after termination with H_2SO_4 (****$p < 0.0001$). **C** Suspension immunofluorescence analysis. *M. synoviae* was treated with anti-MS serum, anti-EF-Ts serum, and preimmune serum, then incubated with a FITC-conjugated goat-anti-rabbit secondary antibody and observed under a fluorescence microscope

Table 2 Mycoplasmacidal rates of anti-EF-Ts serum

Experiment groups	Mean CFU ± SD ($\times 10^3$)	Mycoplasmacidal rates (%)
Rabbit Anti-rEF-Ts serum	265 ± 8.73[a]	33.75 ***[b]
Rabbit anti-*M. synoviae* serum	118 ± 11.38[a]	70.50 ***[b]
Nonimmunized rabbit serum	400 ± 17.33[a]	—
PBS	397 ± 16.21[a]	—

[a] Results were obtained through three replicate experiments

[b] Significant difference compared to nonimmunized rabbit serum according to Student's t-test (***$P < 0.001$)

rEF-Ts stimulate cell proliferation

The role of the rEF-Ts protein after internalization by DF-1 cells was next verified. DF-1 cells were incubated with different concentrations of the rEF-Ts protein, and a CCK-8 kit was used to detect changes in DF-1 cell viability. A rEF-Ts protein concentration greater than 20 μg/ml stimulated cell proliferation (Fig. 8A and B). In addition, when *M. synoviae* was added to DF-1 cells together with rEF-Ts, the reduction in cell viability caused by *M. synoviae* infection was more pronounced than that caused by *M.synoviae* treatment alone (Fig. 8C). However, the specific mechanism involved requires further investigation.

Discussion

An in-depth and systematic understanding of the interactions between *M. synoviae* and host cells is essential for studying the infection mechanism of *M. synoviae* and developing potent vaccines and drugs. The adhesion and colonization of host cells by *Mycoplasma* is a key step in infection. However, *Mycoplasma* species do not contain

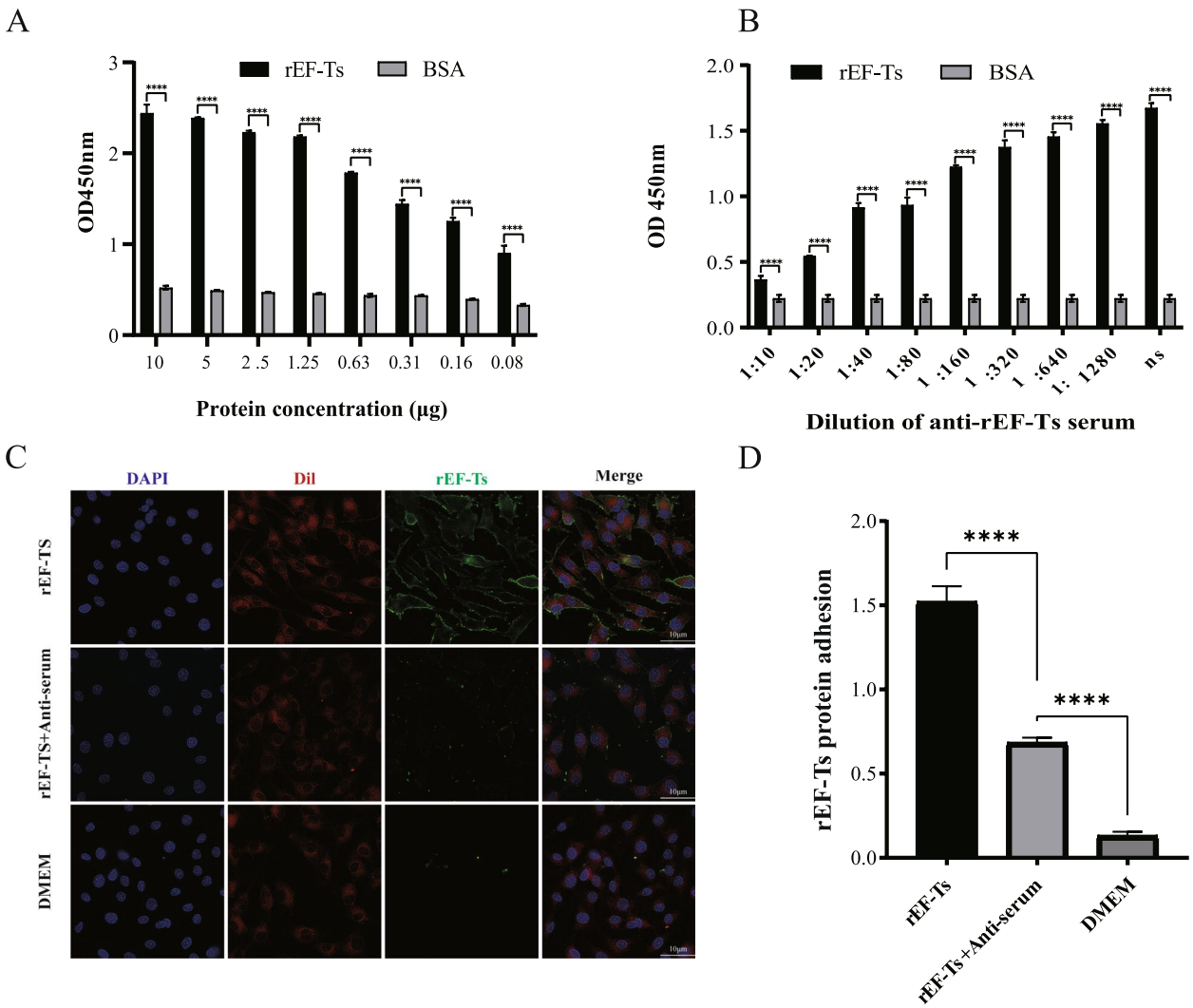

Fig. 3 Binding of *M. synoviae* EF-Ts to DF-1 cell membranes. **A** rEF-Ts bind to DF-1 cell membrane proteins in a dose-dependent manner. Different concentrations of rEF-Ts proteins (0.08 μg–10 μg) were added to a multi-well coated with DF-1 cell membrane protein (0.5 μg). Anti-EF-T serum was used as the primary antibody, an HRP-coupled goat-anti-rabbit antibody was used as the secondary antibody, and the bound rEF-Ts was detected by incubation with tetramethylbenzidine for 10 min, after which the OD was read at 450 nm. Bovine serum albumin was used as the negative control. **B** Anti-EF-T serum inhibited the binding of rEF-Ts to DF-1 cell membrane proteins. rEF-Ts (5 μg) were preincubated with different anti-EF-Ts serum concentrations for 1 h and then added to wells coated with DF-1 cell membrane protein (0.5 μg). Preimmune serum was used as the negative control. **C** Immunofluorescence detection of the effects of rEF-Ts on DF-1 cell adhesion and the inhibition of adhesion induced by anti-EF-Ts serum. The rEF-Ts was pretreated with anti-EF-Ts serum and bound to DF-1 cells for 2 h. After 4% paraformaldehyde fixation, the rEF-Ts was detected with a FITC-labeled goat anti-rabbit secondary antibody. The cells were observed with a laser confocal microscope. Blue indicates nuclei (DAPI), red indicates cell membrane staining (Dil), and green indicates rEF-Ts. **D** Adhesion of the rEF-Ts protein to DF-1 cells was assessed using ImageJ software by the fluorescence intensity (FI) ratios of FITC (green) to DAPI (blue). The values are expressed as the mean ± SD of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

the classical bacterial toxins present in other bacteria; therefore, the virulence molecules used by *M. synoviae* for adhesion, invasion, and immune evasion are not fully defined [11].

In this study, we screened immunogenic membrane proteins of *M. synoviae* by LC–MS/MS and other approaches, revealing that EF-Ts is a virulence factor of *M. synoviae* and may be a secreted protein. There are three main elongation factor types in prokaryotes: elongation factor thermo unstable (EF-Tu), EF-Ts, and elongation factor G (EF-G) [22]. EF-Ts is a guanylate exchange factor for EF-Tu that catalyzes the conversion of GDP complexed with EF-Tu to GTP and the reformation of a dimer of EF-Tu and EF-Ts (EF-T) [32, 33]. EF-Tu

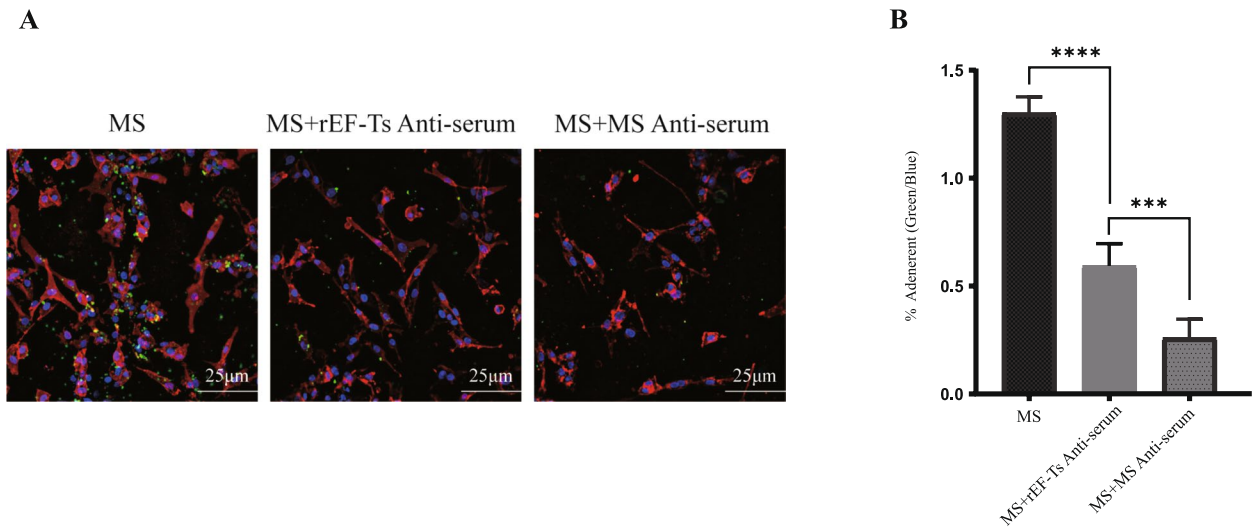

Fig. 4 Antiserum inhibits the adhesion of *M. synoviae* to DF-1 cells. **A** *M. synoviae* was pretreated with anti-EF-Ts serum and anti-*M. synoviae* serum and then incubated with DF-1 cells for 2 h. The cells were observed by laser confocal microscopy. Green represents CFDA-SE-labeled *M. synoviae*, red represents cell membrane staining (Dil), and blue represents cell nuclear staining (DAPI). **B** Antiserum inhibits the adhesion of *M. synoviae* to DF-1 cells, as assessed using ImageJ software. The values are expressed as the mean ± SD of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Table 3 CFU counts in different treatment groups

Experiment groups	Mean CFU ± SD ($\times 10^4$)	Adhesion inhibition rate (%)
Rabbit Anti-rEF-Ts serum	99.6 ± 2.73[a]	84.94***[b]
Rabbit anti-M. synoviae serum	209.7 ± 6.24[a]	74.75***[b]
Nonimmunized rabbit serum	830.6 ± 15.83[a]	—

[a] Results were obtained through three replicate experiments

[b] Significant difference compared to nonimmunized rabbit serum according to Student's t-test (***$P < 0.001$)

has been identified as an important virulence factor in several *Mycoplasma* species and an immunogenic protein in *M. synovi*ae [19, 34, 35]. In the process of investigating the prokaryotic expression of rEF-Ts, it was found that eluting the recombinant protein with different concentrations of imidazole made it difficult to obtain a single target band; instead, two distinct bands were observed, located at 35 KDa and 45 KDa. A literature search led to the development of the hypothesis that the protein band at 45 KDa corresponded to the elongation factor Tu protein in the *E. coli* expression system [31]. The elongation factor Ts tends to form a stable complex, EF-Tu.Ts, with the elongation factor Tu from the *E. coli* expression system during prokaryotic expression [31, 36, 37]. The EF-Tu.Ts complex is highly stable and cannot be dissociated, even in the presence of high concentrations of guanine nucleotides. The EF-Tu.Ts complex was denatured and dissociated using 8 M urea, after which it was purified using Ni–NTA His-Tag purification agarose. Ultimately, a single 37 KDa band was obtained. Subsequently, we showed by western blot and immunofluorescence analyses that EF-Ts is located on the surface of *M. synoviae* cell membranes and is immunogenic, stimulating an immune response in host cells. Numerous studies have demonstrated that EF-Ts is an important virulence factor associated with immune response induction and drug resistance. The expression profile of EF-Ts is elevated in tylosin-resistant *Streptococcus suis* [38], and EF-Ts has been predicted to be involved in drug resistance in *Mycoplasma bovis* [39] and the early immune response in *Helicobacter pylori* [24]. EF-Ts was found to be expressed in different *M. synoviae* isolates with abundant antigenic epitopes; therefore, EF-Ts can be used to screen *M.synoviae* subunit vaccines.

Several studies have shown that *Mycoplasma* adhesion to host cells is a prerequisite for subsequent infection, and that loss of adhesion capacity caused by mutations leads to loss of *Mycoplasma* infectivity, while the restoration of adhesion capacity is often accompanied by the recovery of infectivity and virulence [11, 40, 41]. Mycoplasma cell adhesion is a complex process involving multiple factors. In addition to the major adhesion proteins, several "moonlighting proteins" also participate in the adhesion process of *Mycoplasma* to host cells [11]. Previous studies have shown that *M. synoviae* can adhere to and invade many types of chicken cells [42, 43]. In this study, we performed cellular immunofluorescence visualization and ELISA binding assays to confirm that rEF-Ts

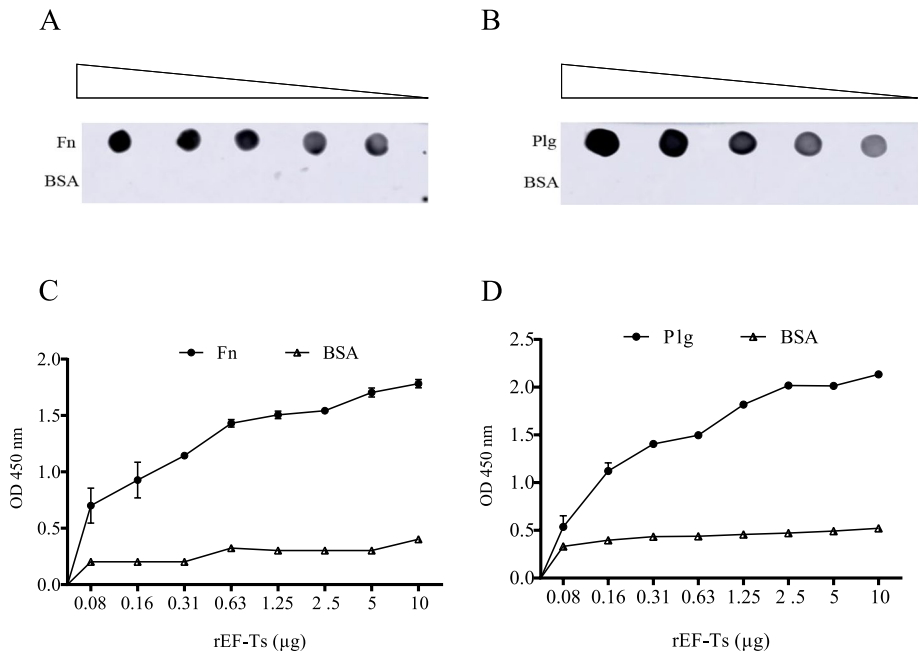

Fig. 5 Binding of *M. synoviae* EF-Ts to Fn and Plg. **A**–**B** The ability of *M. synoviae* EF-Ts to bind fibronectin and plasminogen was determined by dot blotting, with BSA serving as the negative control. **C–D** EF-Ts bind to fibronectin and plasminogen in a dose-dependent manner. Different concentrations of rEF-Ts (0.08–10 μg) were added to wells coated with fibronectin and plasminogen (1 μg). The OD was measured at 450 nm

could adhere to the surface of DF-1 cells and were inhibited by the antiserum. In addition, pretreating DF-1 cells with *M. synoviae* and serum containing rEF-Ts inhibited adhesion and invasion. In conclusion, these studies suggest that EF-Ts are involved in the infection of host cells by *M.synoviae*.

The extracellular matrix (ECM) is an important component of cellular life activities and is involved in building structural scaffolds, regulating physiological processes, cell signaling, and solute migration between tissues and cellular barriers [44]. The ECM is widely distributed and constitutes an important barrier to bacterial and cancer cell migration; it is also a target for adhesion and invasion by various microorganisms [45]. Currently, it is known that various pathogens bind to the ECM via proteins, mediating the adhesion and colonization of host cells. For example, *Leptospira* OmpL47 and LigB are broad-spectrum adhesins that bind to collagen, laminin, fibronectin, elastin, fibrinogen, and others [46]. Fibronectin, an important component of the ECM, is a 440 kDa glycoprotein with multiple structural domains. *Staphylococcus aureus* and *Campylobacter jejuni* binding to fibronectin creates favorable conditions for cell adhesion and invasion [47, 48]. Plasminogen is an important part of the fibrinolytic system. Activated by a plasminogen activator, plasminogen produces a highly efficient serine protease that can hydrolyze fibrin and various ECM components. Numerous invasive pathogens can capture plasminogen and bind to plasminogen receptors (PlgRs) on their surfaces, activating the fibrinolytic system, degrading the ECM system, and facilitating its adhesion and colonization, leading to tissue dissemination [18, 30, 49]. Examples include LppA and LppB in *Mycoplasma bovis* and GAPDH and enolase in *Mycoplasma hyorhinis*, which act as PlgRs to bind and activate plasminogen, enabling the pathogen to degrade the host ECM [28, 30, 50, 51]. This mechanism potentially represents how *Mycoplasma* breaks through the tissue barrier in the host and causes persistent infection in the animal organism. In this study, we found that rEF-Ts can bind Plg and Fn in a dose-dependent manner, suggesting that EF-Ts is potentially a PlgR with important roles in the adhesion and invasion of host cells by *M. synoviae.*

Studies have shown that *M. synoviae* can invade non-phagocytic cells in vitro [43]. Many pathogens invade host cells through clathrin- or caveolin-mediated pathways. For instance, *Edwardsiella tarda* invades cells through clathrin- and caveolin-mediated pathways [52], *Ureaplasma parvum* and *Mycoplasma bovis* are internalized through clathrin-mediated pathways [53, 54], *Mycoplasma gallisepticum* is internalized through cholesterol [55], and *Mycoplasma hyopneumoniae* colonizes porcine epithelial cells through clathrin- and caveolin-mediated pathways [56]. In addition, pathogenic virulence factors, such as the CARDS toxin of *Mycoplasma pneumoniae* [29] and LppB of

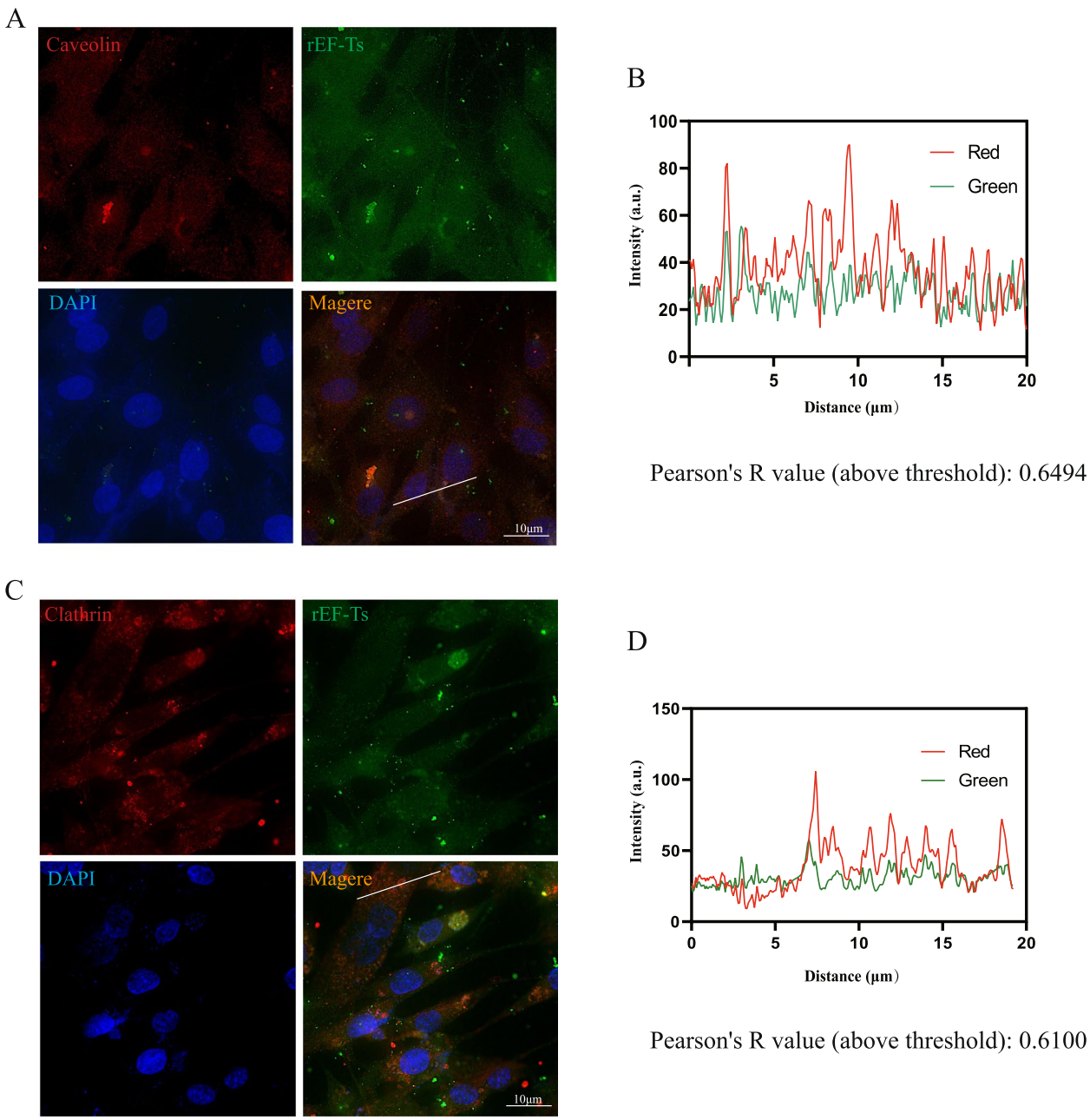

Fig. 6 Colocalization of *M. synoviae* EF-Ts with caveolin-1 (Cav-1) and clathrin heavy chain (CLTC). DF-1 cells were incubated with *M. synoviae* EF-Ts for 2 h and then fixed and labeled with caveolin-1 or clathrin heavy chain (red), rEF-Ts (green), and nuclei (blue). The fluorescence intensity curve and Pearson's R-value were determined by ImageJ software

Mycoplasma bovis [30], can be internalized through the aforementioned pathways. In this study, in addition to degrading the ECM, EF-Ts was internalized by DF-1 cells. Treatment of cells with the endocytosis inhibitors simvastatin and Pitstop-2 significantly reduced the internalization of rEF-Ts. The co-localization of rEF-Ts with clathrin and caveolin was observed by cellular immunofluorescence. In addition, the internalization of rEF-Ts into DF-1 cells promoted cell proliferation. Studies have shown that *M. synoviae* can promote chicken synovial fibroblast cell proliferation [57]. These results suggest that *M.synoviae* EF-Ts is internalized into cells through clathrin- and caveolin-mediated pathways and promotes cell proliferation, providing the conditions necessary for the long-term survival of *M.synoviae* in the host.

In conclusion, we found that the EF-Ts of *M. synoviae* is a multifunctional membrane protein that binds

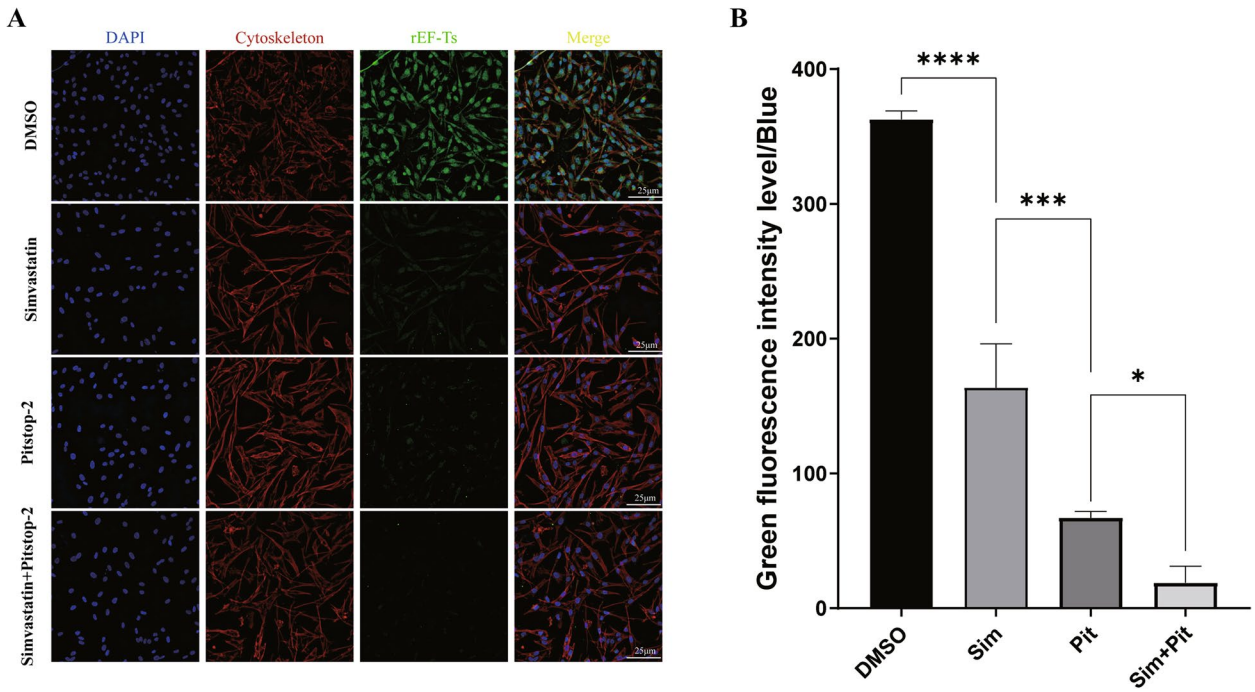

Fig. 7 Internalization of EF-Ts into DF-1 cells via the endocytic pathway. **A** rEF-Ts was incubated for 2 h and then internalized into DF-1 cells. Treatment of cells with clathrin (Pitstop-2, 20 μm) and lipid raft (Simvastatin, 25 μm) inhibitors prevented the internalization of rEF-Ts. DMSO was used as the negative control. Nuclei (blue, DAPI), cytoskeleton (red, phalloidin), rEF-Ts (green), and merged (yellow). **B** Measurement of rEF-Ts (green) fluorescence contrast. The intensity inside each cell after treatment with different inhibitors. The data presented here are the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ indicate significant differences

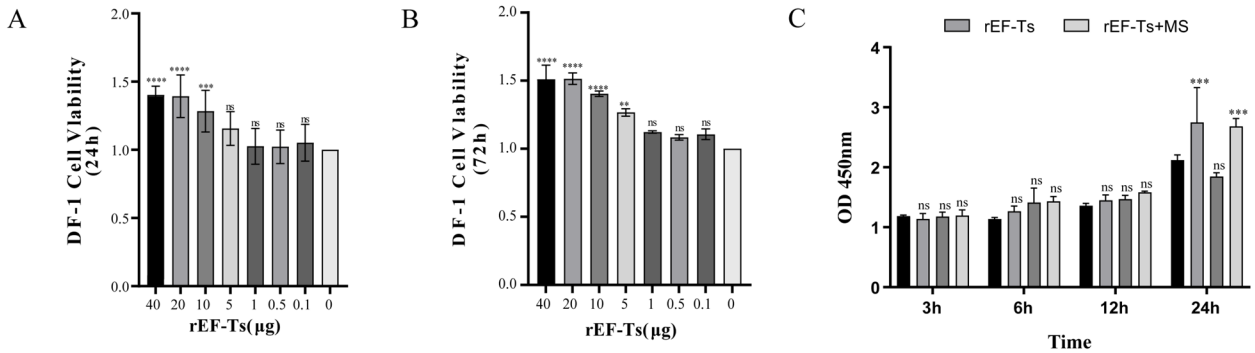

Fig. 8 Effects of rEF-Ts on DF-1 cell proliferation. **A-B** CCK-8 assay showing the effect of incubation with different concentrations of rEF-Ts (0–40 μg) for 24 h or 72 h on the proliferation of DF-1 cells. **C** Treatment with rEF-Ts attenuated the inhibition of DF-1 cell proliferation caused by *M. synoviae* infection. The values are expressed as the means ± standard deviations of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns, no significant difference ($p \geq 0.05$)

to ECM components (Fn and Plg). In this context, EF-Ts could function as an adhesive to help *M. synoviae* adhere to host cell membranes. EF-Ts is internalized into cells through clathrin- and caveolin-mediated pathways and promotes DF-1 cell proliferation. These results deepen our understanding of the role of EF-Ts in the pathogenesis processes of *M. synoviae* and provide new insights for further investigations.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
SB and YZ were involved in the design of the study. YZ, HM and QW performed the experiments. YZ and XH analyzed the data and drafted the manuscript. GQ, XW and XX critically revised the manuscript. All authors read and approved the final manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
New Zealand rabbits were acquired from the Animal Center of Lanzhou Veterinary Research Institute (Lanzhou, China). For the animal experiment, permission from the Animal Care and Ethics Committee of Gansu Agricultural University was obtained (Lanzhou, China), and all methods were carried out in accordance with the relevant guidelines and regulations outlined in the ethics declaration.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Chaidez-Ibarra MA, Velazquez DZ, Enriquez-Verdugo I, Castro Del Campo N, Rodriguez-Gaxiola MA, Montero-Pardo A, Diaz D, Gaxiola SM. Pooled molecular occurrence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in poultry: A systematic review and meta-analysis. Transbound Emerg Dis. 2022;69(5):2499–511.
2. Umar S, Tanweer M, Iqbal M, Shahzad A, Hassan F, Usman M, Sarwar F, Qadir H, Asif S, Un-Nisa Q, et al. RETRACTED: *Mycoplasma synoviae* vaccine modifies virus shedding and immune responses of *Avian influenza* (H9N2) infection in commercial layers. Poult Sci. 2017;96(9):3086–95.
3. Bolha L, Bencina D, Cizelj I, Oven I, Slavec B, Rojs OZ, Narat M. Effect of *Mycoplasma synoviae* and lentogenic *Newcastle disease virus* coinfection on cytokine and chemokine gene expression in chicken embryos. Poult Sci. 2013;92(12):3134–43.
4. Feberwee A, de Wit S, Dijkman R. Clinical expression, epidemiology, and monitoring of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*: an update. Avian Pathol. 2022;51(1):2–18.
5. Landman WJ. Is *Mycoplasma synoviae* outrunning *Mycoplasma gallisepticum*? A viewpoint from the Netherlands. Avian Pathol. 2014;43(1):2–8.
6. Yadav JP, Tomar P, Singh Y, Khurana SK. Insights on *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection in poultry: a systematic review. Anim Biotechnol. 2022;33(7):1711–20.
7. Bergeron N, Hebert G, Pelletier MC, Cai HY, Brochu-Morin ME, Vaillancourt JP. Prevalence of *Mycoplasma synoviae* and Its Impact on Productivity in Commercial Poultry Farms in Quebec. Canada Avian Dis. 2021;65(4):547–53.
8. Cortes V, Sevilla-Navarro S, Garcia C, Tudon A, Marin C, Catala-Gregori P. Seroprevalence and prevalence of *Mycoplasma synoviae* in laying hens and broiler breeders in Spain. Poult Sci. 2021;100(3): 100911.
9. Sun SK, Lin X, Chen F, Wang DA, Lu JP, Qin JP, Luo TR. Epidemiological investigation of *Mycoplasma Synoviae* in native chicken breeds in China. BMC Vet Res. 2017;13(1):115.
10. Zhang X, Chen Y, Xie D, Guo M, Ma S, Chen M, Chu D, Wu Y. Multi-locus sequence typing analysis of *Mycoplasma synoviae* isolates reveals unique sequence types in China. Vet Microbiol. 2021;259: 109101.
11. Dawood A, Algharib SA, Zhao G, Zhu T, Qi M, Delai K, Hao Z, Marawan MA, Shirani I, Guo A. *Mycoplasmas* as Host Pantropic and Specific Pathogens: Clinical Implications, Gene Transfer, Virulence Factors, and Future Perspectives. Front Cell Infect Microbiol. 2022;12: 855731.
12. Bercic RL, Slavec B, Lavric M, Narat M, Bidovec A, Dovc P, Bencina D. Identification of major immunogenic proteins of *Mycoplasma synoviae* isolates. Vet Microbiol. 2008;127(1–2):147–54.
13. Bao S, Guo X, Yu S, Ding J, Tan L, Zhang F, Sun Y, Qiu X, Chen G, Ding C. *Mycoplasma synoviae* enolase is a plasminogen/fibronectin binding protein. BMC Vet Res. 2014;10:223.
14. Hu Z, Li H, Zhao Y, Wang G, Shang Y, Chen Y, Wang S, Tian M, Qi J, Yu S. NADH oxidase of *Mycoplasma synoviae* is a potential diagnostic antigen, plasminogen/fibronectin binding protein and a putative adhesin. BMC Vet Res. 2022;18(1):455.
15. Qi J, Wang Y, Li H, Shang Y, Gao S, Ding C, Liu X, Wang S, Li T, Tian M, et al. *Mycoplasma synoviae* dihydrolipoamide dehydrogenase is an immunogenic fibronectin/plasminogen binding protein and a putative adhesin. Vet Microbiol. 2022;265: 109328.
16. Bao S, Ding X, Yu S, Xing X, Ding C. Characterization of pyruvate dehydrogenase complex E1 alpha and beta subunits of *Mycoplasma synoviae*. Microb Pathog. 2021;155: 104851.
17. Sun Q, Wei X, Chen W, Zhong Q, Yan Z, Zhou Q, Cao Y, Chen F. Characterization and Evaluation of a Novel Conserved Membrane Antigen P35 of *Mycoplasma synoviae*. Front Vet Sci. 2022;9: 836110.
18. Han S, Wang Y, Wang L, Chang W, Wen B, Fang J, Hou X, Qi X, Wang J. *Mycoplasma synoviae* LP78 is a fibronectin/plasminogen binding protein, putative adhesion, and potential diagnostic antigen. Front Microbiol. 2023;14:1335658.
19. Menegatti AC, Tavares CP, Vernal J, Klein CS, Huergo L, Terenzi H. First partial proteome of the poultry pathogen *Mycoplasma synoviae*. Vet Microbiol. 2010;145(1–2):134–41.
20. Zhang G, Han L, Li Z, Chen Y, Li Q, Wang S, Shi H. Screening of immunogenic proteins and evaluation of vaccine candidates against *Mycoplasma synoviae*. NPJ Vaccines. 2023;8(1):121.
21. Zhang G, Han L, Zhao Y, Li Q, Wang S, Shi H. Development and evaluation of a multi-epitope subunit vaccine against Mycoplasma synoviae infection. Int J Biol Macromol. 2023;253(Pt 2):126685.
22. Xu B, Liu L, Song G. Functions and Regulation of Translation Elongation Factors. Front Mol Biosci. 2021;8: 816398.
23. Wang F, Wang X, Ai W, Zeng D, Liang W, Hua L, Liu H, Wang X, Tian Y, Chen H, et al. Three novel immunogenic proteins determined through 2-Dimensional electrophoresis and mass spectrometry with immune serum confer protection against challenge with porcine *Pasteurella multocida* in mouse models. Res Vet Sci. 2021;136:303–9.
24. Seo JH, Youn JH, Kim EA, Jun JS, Park JS, Yeom JS, Lim JY, Woo HO, Youn HS, Ko GH, et al. *Helicobacter pylori* Antigens Inducing Early Immune Response in Infants. J Korean Med Sci. 2017;32(7):1139–46.
25. He Y, Wang H, Chen L. Comparative secretomics reveals novel virulence-associated factors of *Vibrio parahaemolyticus*. Front Microbiol. 2015;6:707.
26. Dawood AS, Zhao G, He Y, et al. Comparative Proteomic Analysis of Secretory Proteins of Mycoplasma bovis and Mycoplasma mycoides subsp. mycoides Investigates Virulence and Discovers Important Diagnostic Biomarkers. Vet Sci. 2023;10(12):685.
27. Zhang H, Lu D, Zhang Y, Zhao G, Raheem A, Chen Y, Chen X, Hu C, Chen H, Yang L, et al. Annexin A2 regulates *Mycoplasma bovis* adhesion and invasion to embryo bovine lung cells affecting molecular expression essential to inflammatory response. Front Immunol. 2022;13: 974006.
28. Liu S, Li Z, Lan S, Hao H, Jin X, Liang J, Baz AA, Yan X, Gao P, Chen S, et al. LppA is a novel plasminogen receptor of *Mycoplasma bovis* that contributes to adhesion by binding the host extracellular matrix and Annexin A2. Vet Res. 2023;54(1):107.
29. Krishnan M, Kannan TR, Baseman JB. *Mycoplasma pneumoniae* CARDS toxin is internalized via clathrin-mediated endocytosis. PLoS ONE. 2013;8(5): e62706.

30. Lan S, Li Z, Hao H, Liu S, Huang Z, Bai Y, Li Y, Yan X, Gao P, Chen S, et al. A genome-wide transposon mutagenesis screening identifies LppB as a key factor associated with *Mycoplasma bovis* colonization and invasion into host cells. FASEB J. 2023;37(10): e23176.
31. Xin H, Leanza K, Spremulli LL. Expression of bovine mitochondrial elongation factor Ts in *Escherichia coli* and characterization of the heterologous complex formed with prokaryotic elongation factor Tu. Biochim Biophys Acta. 1997;1352(1):102–12.
32. Keum M, Ito D, Kim MS, et al. Molecular Effects of Elongation Factor Ts and Trigger Factor on the Unfolding and Aggregation of Elongation Factor Tu Induced by the Prokaryotic Molecular Chaperone Hsp33. Biology (Basel). 2021;10(11):1171.
33. Thirup SS, Van LB, Nielsen TK, Knudsen CR. Structural outline of the detailed mechanism for elongation factor Ts-mediated guanine nucleotide exchange on elongation factor Tu. J Struct Biol. 2015;191(1):10–21.
34. Han S, Wang Y, Chang W, Wang L, Fang J, Han J, Hou X, Qi X, Wang J. Evaluation of the protective efficacy of six major immunogenic proteins of *Mycoplasma Synoviae*. Front Vet Sci. 2023;10:1334638.
35. Yu Y, Wang H, Wang J, Feng Z, Wu M, Liu B, Xin J, Xiong Q, Liu M, Shao G. Elongation Factor Thermo Unstable (EF-Tu) Moonlights as an Adhesin on the Surface of *Mycoplasma hyopneumoniae* by Binding to Fibronectin. Front Microbiol. 2018;9:974.
36. Schwartzbach CJ, Spremulli LL. Bovine mitochondrial protein synthesis elongation factors. Identification and initial characterization of an elongation factor Tu-elongation factor Ts complex. J Biol Chem. 1989;264(32):19125–31.
37. Palmer SO, Rangel EY, Montalvo AE, Tran AT, Ferguson KC, Bullard JM. Cloning and characterization of EF-Tu and EF-Ts from Pseudomonas aeruginosa. Biomed Res Int. 2013;2013:585748.
38. Che RX, Xing XX, Liu X, Qu QW, Chen M, Yu F, Ma JX, Chen XR, Zhou YH, God'Spower BO, et al. Analysis of multidrug resistance in *Streptococcus suis* ATCC 700794 under tylosin stress. Virulence. 2019;10(1):58–67.
39. Bokma J, Vereecke N, Nauwynck H, Haesebrouck F, Theuns S, Pardon B, Boyen F. Genome-Wide Association Study Reveals Genetic Markers for Antimicrobial Resistance in *Mycoplasma bovis*. Microbiol Spectr. 2021;9(2): e0026221.
40. Razin S, Hayflick L. Highlights of mycoplasma research–an historical perspective. Biologicals. 2010;38(2):183–90.
41. Narat M, Bencina D, Kleven SH, Habe F. The hemagglutination-positive phenotype of *Mycoplasma synoviae* induces experimental infectious synovitis in chickens more frequently than does the hemagglutination-negative phenotype. Infect Immun. 1998;66(12):6004–9.
42. Buim MR, Buzinhani M, Yamaguti M, Oliveira RC, Mettifogo E, Ueno PM, Timenetsky J, Santelli GM, Ferreira AJ. *Mycoplasma synoviae* cell invasion: elucidation of the *Mycoplasma* pathogenesis in chicken. Comp Immunol Microbiol Infect Dis. 2011;34(1):41–7.
43. Dusanic D, Bercic RL, Cizelj I, Salmic S, Narat M, Bencina D. *Mycoplasma synoviae* invades non-phagocytic chicken cells in vitro. Vet Microbiol. 2009;138(1–2):114–9.
44. Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK. Extracellular matrix structure. Adv Drug Deliv Rev. 2016;97:4–27.
45. Hynes RO. The extracellular matrix: not just pretty fibrils. Science. 2009;326(5957):1216–9.
46. Daroz BB, Fernandes LGV, Cavenague MF, Kochi LT, Passalia FJ, Takahashi MB, Nascimento Filho EG, Teixeira AF, Nascimento A. A Review on *Host-Leptospira* Interactions: What We Know and Future Expectations. Front Cell Infect Microbiol. 2021;11: 777709.
47. Speziale P, Pietrocola G. The Multivalent Role of Fibronectin-Binding Proteins A and B (FnBPA and FnBPB) of *Staphylococcus aureus* in Host Infections. Front Microbiol. 2020;11:2054.
48. Konkel ME, Talukdar PK, Negretti NM, Klappenbach CM. Taking Control: *Campylobacter jejuni* Binding to Fibronectin Sets the Stage for Cellular Adherence and Invasion. Front Microbiol. 2020;11:564.
49. Law RH, Abu-Ssaydeh D, Whisstock JC. New insights into the structure and function of the plasminogen/plasmin system. Curr Opin Struct Biol. 2013;23(6):836–41.
50. Wang J, Li Y, Pan L, Li J, Yu Y, Liu B, Zubair M, Wei Y, Pillay B, Olaniran AO, et al. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) moonlights as an adhesin in *Mycoplasma hyorhinis* adhesion to epithelial cells as well as a plasminogen receptor mediating extracellular matrix degradation. Vet Res. 2021;52(1):80.
51. Wang J, Yu Y, Li Y, Li S, Wang L, Wei Y, Wu Y, Pillay B, Olaniran AO, Chiliza TE, et al. A multifunctional enolase mediates cytoadhesion and interaction with host plasminogen and fibronectin in *Mycoplasma hyorhinis*. Vet Res. 2022;53(1):26.
52. Sui ZH, Xu H, Wang H, Jiang S, Chi H, Sun L. Intracellular Trafficking Pathways of *Edwardsiella tarda*: From Clathrin- and Caveolin-Mediated Endocytosis to Endosome and Lysosome. Front Cell Infect Microbiol. 2017;7:400.
53. Nishiumi F, Ogawa M, Nakura Y, et al. Intracellular fate of Ureaplasma parvum entrapped by host cellular autophagy. Microbiologyopen. 2017;6(3):e00441.
54. Nishi K, Gondaira S, Fujiki J, Katagata M, Sawada C, Eguchi A, Iwasaki T, Iwano H, Higuchi H. Invasion of *Mycoplasma bovis* into bovine synovial cells utilizing the clathrin-dependent endocytosis pathway. Vet Microbiol. 2021;253: 108956.
55. Furnkranz U, Siebert-Gulle K, Rosengarten R, Szostak MP. Factors influencing the cell adhesion and invasion capacity of *Mycoplasma gallisepticum*. Acta Vet Scand. 2013;55(1):63.
56. Raymond BBA, Turnbull L, Jenkins C, Madhkoor R, Schleicher I, Uphoff CC, Whitchurch CB, Rohde M, Djordjevic SP. *Mycoplasma hyopneumoniae* resides intracellularly within porcine epithelial cells. Sci Rep. 2018;8(1):17697.
57. Liu R, Xu B, Zhang J, Sun H, Liu C, Lu F, Pan Q, Zhang X. *Mycoplasma synoviae* induces serum amyloid A upregulation and promotes chicken synovial fibroblast cell proliferation. Microb Pathog. 2021;154: 104829.

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