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The chaperone GrpE mediates adhesion in *Mycoplasma bovis* and interactions with host extracellular matrix components and plasminogen

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Abstract

Mycoplasma bovis (*M. bovis*) is a significant pathogen responsible for various clinical manifestations in cattle, including pneumonia, mastitis, and arthritis. The challenges associated with managing *M. bovis* infections include the absence of specific therapeutic agents, increasing antibiotic resistance and the absence of an effective commercial vaccine. The chaperone GrpE is a crucial component of mycoplasma bacteria. However, because there are few tools for genetic manipulation of *M. bovis*, the role of GrpE in pathogenicity is yet unknown. In this study, GrpE was universally present in the tested *M. bovis* strains, and it was localized on the surface membrane. We first demonstrated that GrpE plays a vital role in the ability of *M. bovis* to adhere to embryonic bovine lung (EBL) cells in a dose-dependent manner. Importantly, the presence of anti-GrpE serum specifically inhibited this adhesion. Additionally, GrpE was shown to bind various components of the host extracellular matrix (ECM), such as fibronectin, collagen IV, laminin, and vitronectin. This interaction not only facilitated adhesion but also promoted the activation of plasminogen into plasmin via tissue plasminogen activator (tPA). The significance of GrpE was further underscored in that disruption of the *grpE* gene markedly reduced the adhesion of *M. bovis* to EBL cells, whereas this ability was restored in the complemented strain *M. bovis* Δ *grpE:grpE*. In conclusion, GrpE represents a novel adhesion protein in *M. bovis* that is crucial for its interaction with the host ECM and plasminogen. This research highlights the importance of GrpE in the pathogenicity of *M. bovis* and suggests that targeting this protein could offer new avenues for vaccine development and therapeutic strategies against *M. bovis* infections.

Keywords GrpE, *Mycoplasma*, extracellular matrix, plasminogen

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Introduction

Mycoplasma bovis (*M. bovis*) is a globally prevalent pathogen that significantly affects the cattle industry and has serious economic repercussions. *M. bovis* poses a substantial threat to livestock health and is the primary cause of chronic pneumonia, arthritis, mastitis, otitis media, and meningitis in cattle [1, 2]. Its lack of a cell wall makes it resistant to antibiotics that target cell wall synthesis, rendering those treatment options ineffective. Consequently, there is an urgent need for effective antibiotics and a demand for viable disease control strategies [3, 4]. There are no effective commercial vaccines available for managing *M. bovis* infections, underscoring the need for intensified research efforts to combat this pathogen [5, 6].

As a small cell wall-deficient bacterium, *M. bovis* has unique structural characteristics that facilitate direct interactions with the membrane components of host cells. This property is pivotal for cytoadherence, which serves as a critical initial step in the colonization process and significantly contributes to the pathogenicity of mycoplasmas. This selective adhesion is vital for bacterial survival and colonization within the host environment. To achieve effective adherence and colonization, *M. bovis* expresses a range of adhesins, which latch onto extracellular matrix (ECM) components and plasminogen. Some of the adhesins identified in *M. bovis* include methylenetetrahydrofolate tRNA-(uracil-5-)-methyltransferase (TrmFO) [7], fructose-1,6-bisphosphate aldolase (FBA) [8, 9], α -enolase [10], mbfN [11], *Mycoplasma* immunogenic lipase A (MilA) [12], Mbov0503 [13] and lipoprotein LppA [14]. These adhesins, especially membrane proteins, may interact with various ECM components and plasminogen (Plg), thereby mediating cytoadherence. This interaction not only promotes the adhesion and invasion of pathogenic bacteria into host cells but is also involved in activating the immune response [14].

Molecular chaperones, commonly referred to as heat shock proteins (HSPs), have been identified in various bacterial species [15, 16]. GrpE is recognized as the sole nucleotide exchange factor for Hsp70 and organelles derived from bacteria and plays a crucial role in bacterial pathogenicity [17, 18]. Structurally, GrpE is characterized by an α -helix dimerization domain and a β -domain that facilitates its interaction with DnaK [17]. GrpE is involved in the adhesion and pathogenicity of group A *Streptococcus* (GAS) and *Streptococcus suis* [19, 20]. Moreover, there is a correlation between GrpE expression and stress conditions during the biofilm formation of certain bacteria [21–23]. Upon entering the host, bacteria are immediately exposed to different environments, including fluctuations in temperature, osmotic pressure, and pH. To effectively adhere, invade, or counteract these stressors, bacteria upregulate the expression

of virulence factors and stress response proteins, which include HSPs. These are actively employed by bacteria to mount a robust compensatory response to adverse conditions, thereby ensuring their survival and pathogenicity [19, 24].

Preventive vaccines necessitate the expression of target antigens during the initial stages of infection, enabling recognition by the host immune system [25, 26]. GrpE is expressed by the immune system early in the infection process and provides protective immunity and substantial protection against *Mycobacterium tuberculosis* [26]. Furthermore, it presents a promising antigen target for the formulation of multiantigenic vaccines aimed at combating *Ureaplasma urealyticum* [27]. Importantly, the lack of efficient genetic tools for *Mycoplasma* results in a limited understanding of the function of GrpE in pathogenicity and the immune response. Therefore, the aim of this study was to investigate the potential mechanisms through which GrpE influences the adhesion of *M. bovis*, thereby enhancing our understanding of its pathogenicity. Understanding these mechanisms is crucial, as it provides important insights into how *M. bovis* establishes infection, which has significant implications for animal health. Furthermore, the findings from this research may position GrpE as a promising candidate for vaccine development, offering a strategic approach to prevent *M. bovis* infections.

Materials and methods

Strains and cells

M. bovis reference strain PG45, along with 6 clinical *M. bovis* isolates (Additional file 1), were cultivated in modified pleuropneumonia-like organisms (PPLO) medium at 37 °C, following previously established protocols [14]. *M. bovis* Δ grpE, identified from the transposon mutant library as previously described [28], was grown in modified PPLO medium supplemented with kanamycin (100 μ g/mL). To establish the complementation strain *M. bovis* Δ grpE:grpE, the recombinant plasmid pIRR45-grpE was introduced into the Δ grpE mutant using the polyethylene glycol (PEG) method as previously described [28] and maintained in medium supplemented with 5 μ g/mL tetracycline. DNA cloning and protein expression were conducted using *Escherichia coli* (*E. coli*) strains DH5 α and BL21(DE3) (TransGen Biotech, China), respectively. Embryonic bovine lung (EBL) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and incubated at 37 °C with 5% CO₂. The following day, the EBL cells were observed under a microscope (Olympus CKX31, Japan) for further experiments.

DNA constructs

The pIRR45-*grpE* plasmid was constructed using an In-Fusion kit (Takara Bio, Japan) following the manufacturer's guidelines as previously outlined [28]. The *grpE* gene was amplified with the primers pIRR45-*grpE*-F/R (5'-tggactagtgcggccATGAGTTCGATGAATAAAGAA GAAG-3'; 5'-gacctgcaggcggccTTACTTATTGCTTTT TGCAATTGATTC-3') and subsequently ligated to the complementary plasmid pIRR45 (pIRR45-F: 5'-GGC CGCCTGCAGGTCGACCATAT-3'; pIRR45-R: 5'-GCA CTAGTCCAGATTATATAACAAC-3') via an In-Fusion kit. This construct was subsequently transformed into *E. coli* DH5 α and subsequently verified by PCR.

Bioinformatics analysis

Amino acid sequences that were homologous or similar to those of the GrpE protein in *Mycoplasma* were screened in the NCBI database by using the BLASTP program with default parameters. Sequence alignment was subsequently performed by the Clustal W method, and a phylogenetic tree was constructed using the neighbour-joining (NJ) method with 1000 bootstraps in MEGA 11 software.

Recombinant protein expression and polyclonal antibody preparation

To express the recombinant proteins, the pET-30a-*grpE* plasmid was introduced into *E. coli* BL21 (DE3) cells. Following transformation, protein expression was induced using isopropyl β -D-thiogalactoside (IPTG, 0.05 mM), which was conducted in a shaking incubator at 16 °C for 16 h. The resulting protein was subsequently purified utilizing nickel-charged resin Ni-NTA (GenScript Biotech Corporation, China). The concentration of the purified protein was quantified using a bicinchoninic acid (BCA) assay kit (Beyotime, China). For polyclonal antibody production, the purified protein rGrpE was combined at a 1:1 (v/v) ratio with the adjuvant QuickAntibody-Mouse 5W (Biodragon, China) and administered intramuscularly to 6-week-old BALB/c mice at an interval of 21 days between two immunizations. Antisera were collected on day 35, the antibody titre was assessed via enzyme-linked immunosorbent assay (ELISA), and the specificity of the generated polyclonal antibody was evaluated through western blot analysis.

Localization of GrpE in *M. bovis*

To determine the localization of GrpE, cytoplasmic and cell membrane proteins from *M. bovis* PG45 were extracted using a membrane extraction protein kit (Thermo Fisher, USA). The proteins, including whole bacterial protein, cell membrane protein, and cytoplasmic protein, were subsequently separated via 10%

SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham, USA). Afterwards, the membrane was blocked with 10% healthy goat serum (ZSGB-BIO, China) at room temperature for 2 h, after which it was incubated with anti-GrpE serum as the primary antibody (1:10 000) for 2 h at room temperature. The membrane was then washed 4 times with Tris-buffered saline Tween (TBST) and incubated for 1 h at room temperature with goat anti-mouse HRP-IgG (1:1000; Beyotime, China) as a secondary antibody. Imaging was conducted using a ChemiDoc imaging system (Bio-Rad, USA).

Binding of EBL cell membrane protein detected by ELISA

EBL cell membrane protein was extracted utilizing a commercial cell membrane extraction kit (Thermo Fisher Scientific) following the manufacturer's instructions. ELISA was performed as previously described [28], with some modifications; a 96-well plate was coated with 800 ng of EBL cell membrane protein and subsequently blocked with 5% skim milk powder at 37 °C for 2 h. Various concentrations of rGrpE (800 ng to 3.125 ng) or rGrpE (800 ng) that had been preincubated with different dilutions of anti-GrpE serum or negative mouse serum from 1:10 to 1:2560 were then introduced and incubated at 37 °C for 1 h. Mouse anti-GrpE serum served as the primary antibody (1:1000 dilution), while goat anti-mouse HRP-IgG functioned as the secondary antibody (1:1000 dilution). Following 1 h of incubation at 37 °C, the OD was measured at a wavelength of 450 nm using an iMark microplate reader (Bio-Rad).

Dot blot analysis

Twofold-diluted rGrpE protein and 6 $\times$ His polypeptides were applied to a nitrocellulose membrane (Solarbio, China) and allowed to dry at room temperature for 1 h. The membranes were subsequently subjected to blocking buffer (Beyotime, China) at room temperature for 1 h. Subsequently, the membranes were incubated overnight at 4 °C on a shaking platform with 10 μ g of fibronectin (Sigma-Aldrich), collagen IV (Sigma-Aldrich), laminin (Roche), vitronectin (Sigma-Aldrich), tissue-type plasminogen activator (tPA), or plasminogen (Cell Sciences). After incubation, the membranes were washed 5 times with TBST for 10 min each. Next, antibodies against fibronectin, collagen IV, laminin, vitronectin, tPA, or plasminogen (1:500; Abcam) were added, and the membranes were incubated at room temperature for 1 h. After another wash with TBST, either goat anti-mouse IgG-HRP (1:1000; Abcam) or goat anti-rabbit IgG-HRP (1:5000; Abcam) was added, and the membranes were incubated at room temperature for 1 h. Signal detection

was carried out using ECL (Thermo Fisher Scientific) on an imaging detection system (Bio-Rad).

ELISA for determination of ECM components

Fibronectin, collagen IV, laminin, and vitronectin (100 ng each) were added to 96-well plates. Subsequently, rGrpE protein was applied to the ELISA plates, which had been precoated with the ECM proteins, at various dilutions ranging from 800 ng to 3.125 ng, as previously outlined [14]. OD readings were recorded at a wavelength of 450 nm using an iMark microplate reader (Bio-Rad).

Plasminogen activation assay

The rGrpE protein (20 µg/mL) was incubated with plasminogen (20 µg/mL) at 37 °C for 1 h. Subsequently, the mixture was transferred to a 96-well plate containing tPA (50 ng/mL, Sigma) or the lysine analogue aminoacetic acid ϵ -ACA (80 mM, Sigma), followed by an additional incubation at 37 °C for 15 min. After an incubation period, the D-Val-Leu-Lys para-nitroaniline hydrochloride substrate (0.5 mM, Sigma) was added, and the OD was monitored at 405 nm at 15 min intervals using an iMark microplate reader (Bio-Rad).

Adherence and inhibition assays of rGrpE

rGrpE or 6×His peptides (100 µg) were added to Opti-MEM (Gibco) and incubated overnight with EBL cells in a 24-well plate at 37 °C for 1 h. The EBL cells were subsequently fixed using 4% paraformaldehyde (PFA; Solarbio, China) at room temperature for 10 min, permeabilized with 0.1% Triton X-100 (Solarbio, China) for an additional 10 min, and blocked with 5% bovine serum albumin (BSA; Sigma) at 37 °C for 2 h. In the adhesion inhibition experiment, 100 µg of rGrpE protein was combined with mouse anti-GrpE serum or negative mouse serum (10 µL) in Opti-MEM and incubated at 37 °C for 1 h before the cells were fixed. Following a wash with phosphate-buffered saline (PBS), the cells were incubated with anti-GrpE serum or negative mouse serum (1:100) at 37 °C for 3 h and subsequently treated with goat anti-mouse Dylight 488 (1:1000; Thermo Fisher) for 45 min at 37 °C. EBL cell membranes and nuclei were stained with DiI (Beyotime, China) and 4',6-diamidino-2-phenylindole (DAPI, Sigma), respectively. The cells were then observed with a Zeiss LSM 980 inverted laser scanning confocal microscope.

Mycoplasma adhesion assay

EBL cells were cultured in 12-well plates and incubated overnight at 37 °C in a CO₂ incubator. The cells were subsequently infected with *M. bovis* at a multiplicity of infection (MOI) of 1000 for 1.5 h at 37 °C. Following the infection period, the cells were washed 3 times with PBS

and digested using TrypLE™ Express (Gibco), and the resulting dilutions were plated onto PPLO agar plates and then incubated at 37 °C with 5% CO₂. The number of *M. bovis* colonies was counted after 3 days of incubation, and the adhesion percentage was calculated. In the adhesion inhibition experiment, anti-GrpE serum was preincubated with *M. bovis* at ratios of 1:10 and 1:20 at 37 °C for 1 h, while preimmune serum was used as a negative control.

Statistical analysis

All the statistical analyses were conducted using unpaired *t* tests and one-way/two-way analysis of variance with GraphPad Prism 9.0 software. All values are expressed as the means ± standard deviations. Three independent experiments were conducted. Significant differences are indicated as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and ns, not significant at *p* ≥ 0.05.

Results

Bioinformatics analysis

Genomic analysis revealed that the *grpE* gene is ubiquitously present across *M. bovis* isolates and represents one of the core genes of this species. The GrpE protein sequences among various *M. bovis* strains exhibited highly conserved amino acid sequences, with only a small number of mutations observed throughout the complete open reading frame, such as S112L, N119K, and L313S, which are present in very few isolates (Figure 1A). Moreover, according to phylogenetic analysis, GrpE can be categorized into five groups on the basis of its amino acid sequence in *Mycoplasma* species. *M. bovis* and *Mycoplasma agalactiae* are closely clustered together, and *Mycoplasma primatum*, *Mycoplasma felifaucium* and *Mycoplasma maculosa* form a large branch (Figure 1B). Multiple sequence alignment analysis revealed multiple conserved amino acid residues in GrpE. Specifically, the 174–298 aa region of the GrpE protein, along with residues Q188, P248, R288, and V295, was significantly conserved among *Mycoplasma* species (Figure 1C). These conserved sites may have comparable functional roles in diverse *Mycoplasma* species.

Expression and purification of rGrpE

The recombinant expression plasmid pET30a-*grpE* was successfully constructed. Following induction with 0.05 mM IPTG for 16 h, the rGrpE protein was subsequently purified using Ni-NTA and predominantly expressed as a soluble protein of approximately 46 kDa in recombinant *E. coli* BL21(DE3), as confirmed by SDS-PAGE analysis (Additional file 2). Moreover, western blot analysis indicated that purified rGrpE was effectively

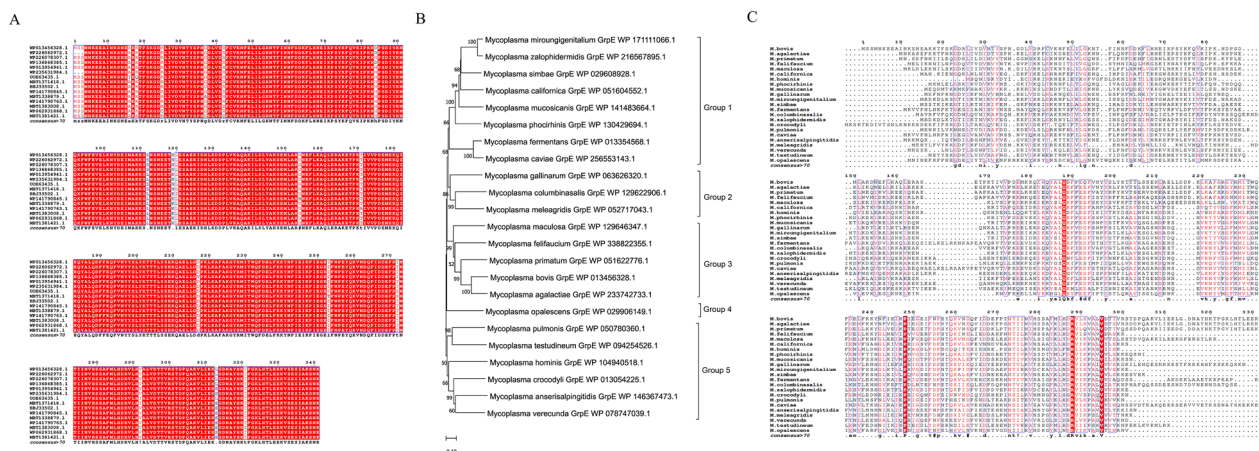


Figure 1 Bioinformatics analysis of GrpE in *Mycoplasma* species. A Genetic characteristics of *grpE* in *Mycoplasma bovis*. **B** Genetic characteristics of *grpE* in *Mycoplasma* species. A phylogenetic tree based on GrpE protein sequences was constructed using the neighbour-joining (NJ) method with 1000 bootstraps in MEGA 11 software. The phylogenetic tree was divided into five groups. **C** Multiple conserved sites of GrpE in *Mycoplasma* based on multiple alignment. Highly conserved residues are shown in red.

recognized by mouse anti-6×His monoclonal antibodies and anti-GrpE serum (Additional file 1).

Surface localization of *M. bovis* GrpE and its adhesion to EBL cells

Western blot analysis confirmed the expression of GrpE among *M. bovis* PG45 and other clinical isolates (Figure 2A, Additional file 2). The localization of GrpE in the *M. bovis* PG45 strain was assessed through analysis of membrane proteins, cytoplasmic proteins, and whole-cell proteins in anti-GrpE serum. The results indicated that GrpE was predominantly localized in the membrane fraction of *M. bovis* (Figure 2B). This distribution highlighted the potential role of GrpE as a membrane-associated protein in the pathogenicity of *M. bovis*. Confocal microscopy revealed that rGrpE had a strong affinity for EBL cells, with a marked decrease in adhesion noted when rGrpE was pretreated with anti-GrpE serum, indicating that this serum significantly disrupted the binding of rGrpE, while the application of negative mouse serum did not significantly inhibit the adhesion process (Figure 2C). The 6×His peptides did not adhere to EBL cells (Figure 2C). These findings underscored the specific interaction between GrpE and EBL cells. The binding interaction between GrpE and membrane extracts derived from EBL cells was examined using ELISA. Compared with the control group, the rGrpE-treated group exhibited dose-dependent binding affinity to the EBL cell membrane (Figure 2D). Furthermore, the binding of rGrpE to the EBL cell membrane was effectively inhibited by anti-GrpE serum at a dilution of 1:10 to 1:640, while the inhibitory effect of negative serum was not

significant, suggesting that the interaction was specific and can be modulated by antibodies targeting GrpE.

GrpE binds with host ECM components

To further investigate the adhesion properties of GrpE to host cells, we examined its potential interactions with ECM components. We employed both dot blot analysis and ELISA to evaluate the binding affinity of rGrpE for fibronectin, collagen IV, laminin, and vitronectin, with 6×His peptides serving as a negative control. The findings confirmed that GrpE bound to fibronectin, collagen IV, laminin, and vitronectin in a dose-dependent manner (Figures 3A–H). This clear pattern of binding highlights the potential role of GrpE in mediating interactions with host cells.

GrpE promotes the conversion of plasminogen to plasmin

GrpE exhibited a robust ability to bind plasminogen and tPA in a dose-dependent manner, as demonstrated by dot blot analysis (Figures 4A, B). The binding of GrpE to plasminogen by tPA was assessed using plasminase-specific chromogenic substrates, which allowed us to quantitatively measure plasminogen activation. The results indicated that wells containing rGrpE, plasminogen, and tPA displayed significantly higher OD readings at 405 nm than control wells containing 6×His peptides did (Figure 4C). This increase underscores the effective conversion of plasminogen to plasminase facilitated by GrpE in the presence of tPA. Moreover, the addition of ε-ACA inhibited the capacity of GrpE to promote plasminogen activation, further supporting the specificity of the interaction between GrpE and plasminogen activation.

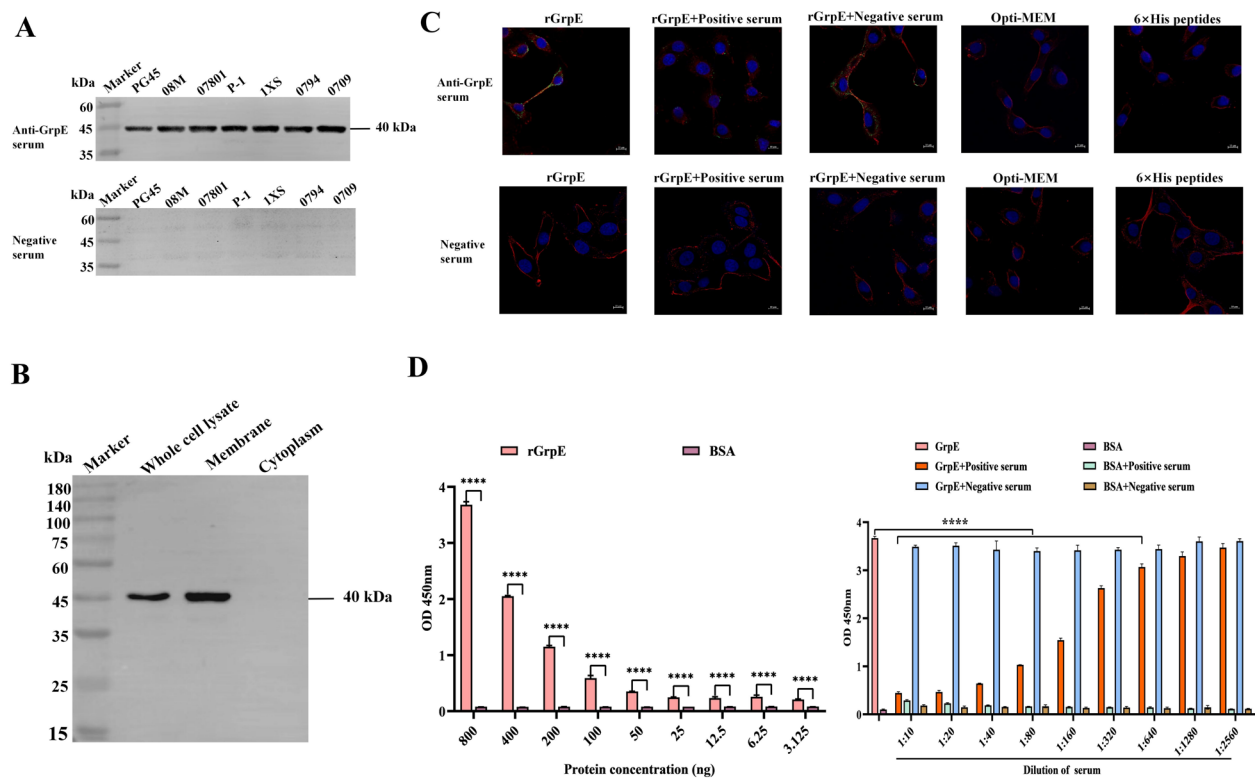


Figure 2 Surface localization of GrpE in *M. bovis* and its adhesion to EBL cells. **A** Evaluation of GrpE immunogenicity across different *M. bovis* strains. Whole-cell proteins from seven *M. bovis* strains were transferred to PVDF membranes, incubated with anti-GrpE serum or negative mouse serum (1:10 000), and subsequently detected using goat anti-mouse IgG-HRP (1:1000). **B** Membrane and cytoplasmic proteins of *M. bovis* PG45 were extracted, incubated with anti-GrpE serum (1:10 000), and then probed with goat anti-mouse HRP-IgG. **C** The adhesion of GrpE to EBL cells was observed by laser confocal microscopy. One hundred micrograms of rGrpE (6×His peptides) was preincubated with positive or negative serum for 1 h and then tested with anti-GrpE serum or negative mouse serum (10 μL) and goat anti-mouse Dylight 488 (green, 1:1000). The cell membrane (red) and nucleus (blue) were labelled with Dil and DAPI, respectively (scale bar = 10 μm). **D** The binding of *M. bovis* GrpE and EBL cell membranes detected by ELISA. The EBL membrane protein (800 ng) was coated overnight and subsequently incubated with varying concentrations of rGrpE (ranging from 800 ng to 3.125 ng) for 1 h, after which anti-GrpE serum (1:1000) and goat anti-mouse IgG-HRP (1:1000) were added for TMB detection. For the inhibition test, EBL cell membrane protein (800 ng) was coated overnight, and rGrpE (800 ng) was incubated with anti-GrpE serum at different dilutions for 1 h. Subsequently, anti-GrpE serum (1:1000) and goat anti-mouse IgG-HRP (1:1000) were added for TMB detection. The values are presented as the mean ± standard error of three replicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Disruption of the *grpE* gene significantly reduces *M. bovis* adhesion to host cells

The complementary strain *M. bovis* Δ *grpE:grpE* was successfully developed using the pIRR45-*grpE* complementary plasmid and identified by PCR (Additional file 3). Western blot analysis confirmed the expression of GrpE in the complementary strain *M. bovis* Δ *grpE:grpE* and the wild-type strain, whereas it was absent in the *M. bovis* Δ *grpE* strain (Figure 5A). Compared with those of the wild-type strain *M. bovis* PG45, the growth curves of these strains revealed that the lack of the *grpE* gene had a minimal effect on the growth of *M. bovis* (Figure 5B). However, the adhesion of *M. bovis* Δ *grpE* to EBL cells significantly decreased, whereas the adhesion of the complemented strain *M. bovis* Δ *grpE:grpE* exhibited a notable restoration of adhesion (Figure 5C). This finding suggests that the disruption of

grpE gene adversely affects the adhesion of *M. bovis* to EBL cells. Additionally, these observations were corroborated by laser confocal microscopy analysis (Figure 5E). Before EBL cells were infected with *M. bovis*, anti-GrpE serum was incubated with *M. bovis*. The results demonstrated that compared with pretreatment with preimmune serum, treatment with anti-GrpE serum led to a significant reduction in the adhesion of *M. bovis* to EBL cells (Figure 5D). Importantly, the inhibitory effect of anti-GrpE serum was concentration dependent, indicating that higher concentrations of serum corresponded to greater reductions in bacterial adhesion. Taken together, these findings suggest that GrpE plays a vital role in the adhesion of *M. bovis* to EBL cells.

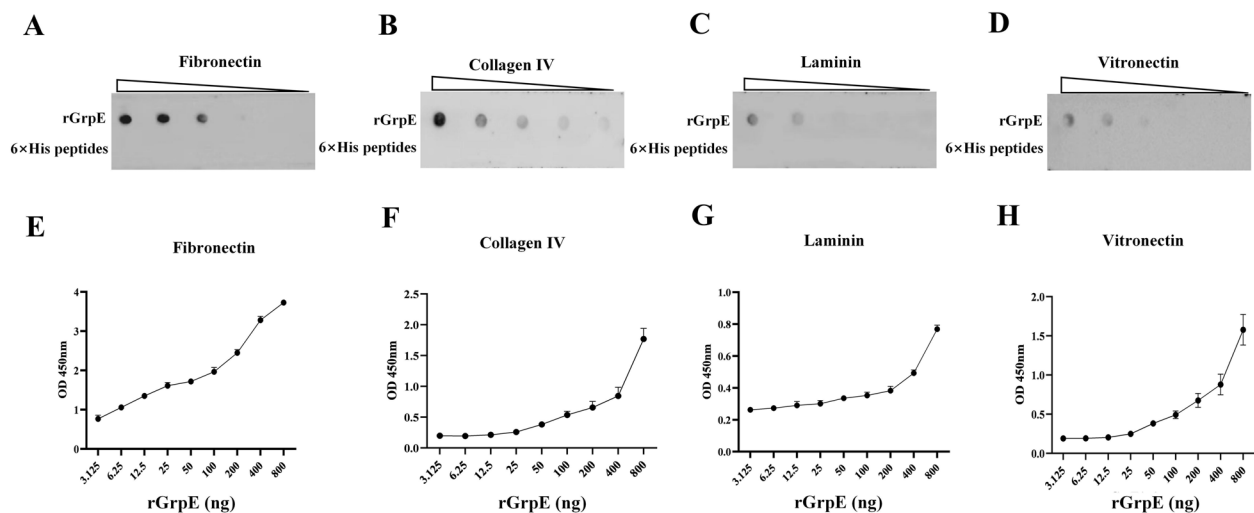


Figure 3 Interaction of rGrpE with host extracellular matrix components. **A–D** The detection of rGrpE binding to various host ECM components, including fibronectin, collagen IV, laminin, and vitronectin, using a dot blot assay, with a 6×His peptides serving as the negative control. **E–H** The binding of rGrpE to fibronectin, collagen IV, laminin and vitronectin in a dose-dependent manner, as evidenced by ELISA.

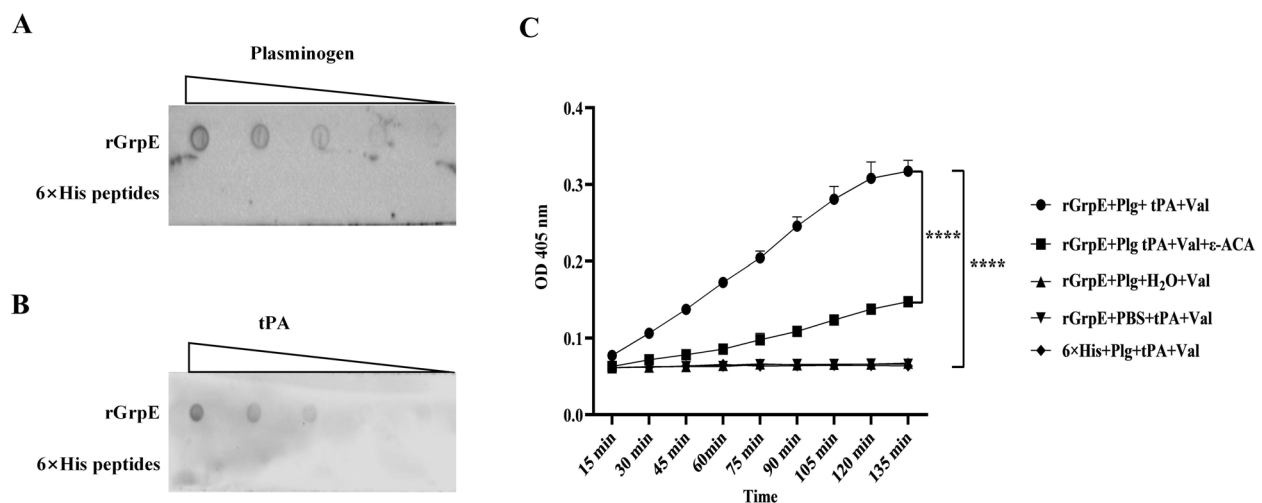


Figure 4 Plasminogen activation activity mediated by GrpE. **A, B** The binding affinity of rGrpE for both plasminogen and tPA, as assessed by dot blot analysis, with 6×His peptides serving as a negative control. **C** GrpE facilitated plasminogen to plasmin in the presence of tPA. rGrpE was incubated with plasminogen for 1 h, tPA and the specific substrate d-Val-Leu-Lys p-nitroaniline dihydrochloride were added, and plasmin activity was measured as the optical density at 405 nm at 15 min intervals. ε-ACA was included as a lysine analogue.

Discussion

Adhesion to host cells is the first step in many mycoplasma infections. The binding of mycoplasma to host cells is essential for the initiation of infection and subsequent colonization [14]. Owing to their obligate parasitic lifestyle, the surface lipoproteins and various membrane-related proteins of mycoplasma play critical roles in adhering to host tissues, which are intricately linked to the pathogenesis of these organisms and are often regarded as the main candidate antigens for vaccine

development [11, 29]. GrpE is highly conserved, which implies that it may be critical for the biological functions of mycoplasmas. In this study, we examined the role of GrpE in adhesion. GrpE is primarily associated with the membrane of *M. bovis*, and this surface localization suggests that it may be involved in crucial interactions with host cell membranes, potentially facilitating bacterial adhesion and colonization. By confocal microscopy analysis, our study revealed the adhesion of rGrpE to EBL cells and a significant reduction in adhesion following

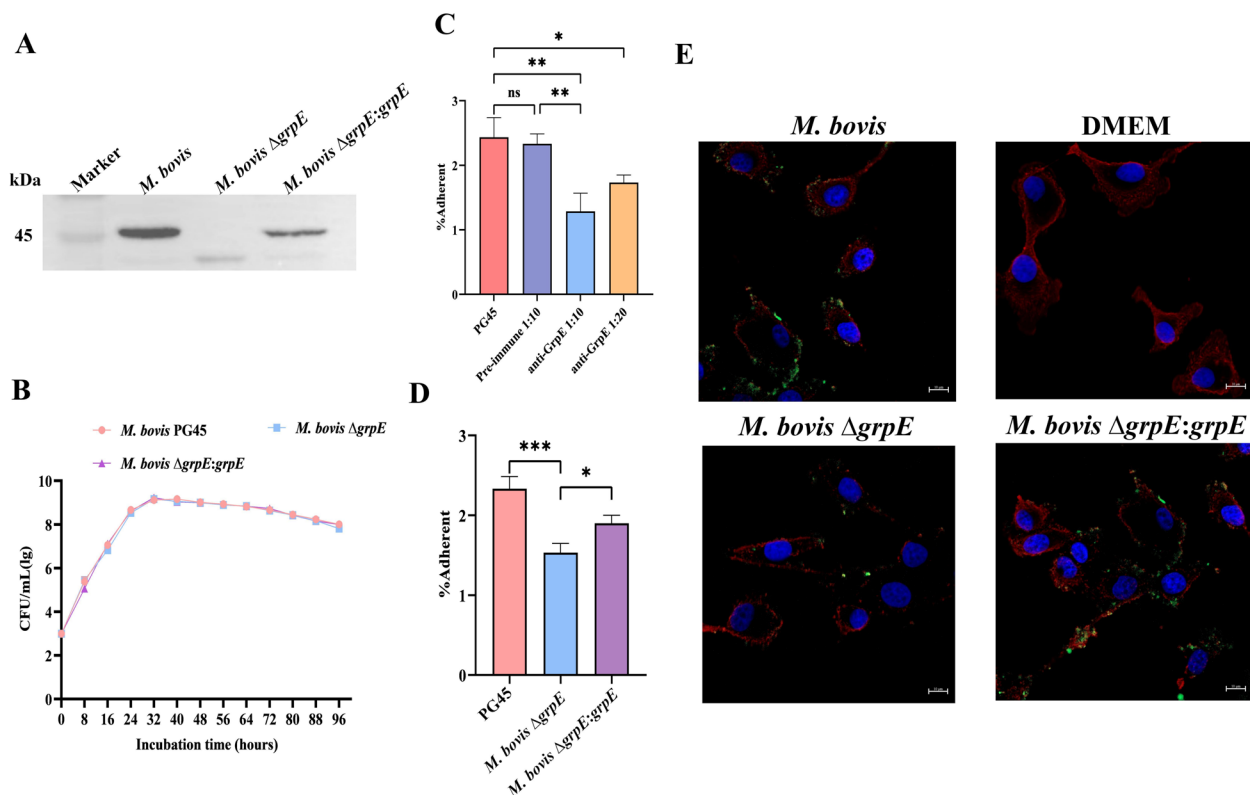


Figure 5 Adhesion ability of GrpE. **A** The expression of the GrpE protein in the *M. bovis* wild-type strain, *M. bovis* Δ grpE strain, and *M. bovis* Δ grpE:grpE strain detected by anti-GrpE serum. **B** Growth curves of the *M. bovis* wild-type strain PG45, *M. bovis* Δ grpE, and *M. bovis* Δ grpE:grpE. **C** The ability of *M. bovis* wild-type, *M. bovis* Δ grpE, and *M. bovis* Δ grpE:grpE strains to adhere to EBL cells was determined 1.5 h after infection, after which the cells were washed three times with PBS, digested with TrypLE™ Express, diluted and placed on mycoplasma agar plates for counting. **D** Inhibition of adhesion to *M. bovis* by GrpE. *M. bovis* was incubated with different concentrations of anti-GrpE serum (1:10, 1:20) at 37 °C for 1 h, after which the EBL cells were infected for 1.5 h. Preimmunized serum served as a control. **E** The adhesion ability of *M. bovis* wild-type, *M. bovis* Δ grpE, and *M. bovis* Δ grpE:grpE to EBL cells was observed by laser scanning confocal microscopy. *M. bovis* wild-type, *M. bovis* Δ grpE, and *M. bovis* Δ grpE:grpE were infected into EBL cells, followed by staining with CFDA-SE, with the nuclei labelled using DAPI and the cell membrane marked with Dil. Red represents the cell membrane, green denotes *M. bovis*, and blue represents the nucleus.

pretreatment with anti-GrpE serum, suggesting that GrpE plays a pivotal role in mediating the adhesion of *M. bovis* to host cells. These findings were further supported by the ELISA analysis, confirming that rGrpE binds to EBL cell membranes in a dose-dependent manner. Moreover, the function of GrpE in cytoadhesion at the bacterial level was investigated, and the results revealed that disruption of the *grpE* gene markedly impaired the adhesion of *M. bovis* to EBL cells, whereas the complemented strain *M. bovis* Δ grpE:grpE restored this ability, confirming that GrpE is indispensable for bacterial adhesion. Furthermore, treatment with anti-GrpE serum significantly inhibited the adhesion process of *M. bovis* in a concentration-dependent manner. Collectively, these results indicate that GrpE is a novel adhesion protein in *M. bovis*.

The host ECM is crucial for cell adhesion, and pathogenic microorganisms exploit interactions to facilitate their colonization on host cell surfaces, leading to

infection [28, 30]. The interaction of rGrpE with ECM components, such as fibronectin, collagen IV, laminin, and vitronectin, was dose dependent, which indicated the multifaceted role of rGrpE in facilitating adherence to host cells and enhancing the survival and persistence of *M. bovis* within the host, which aligns with previous data that support the idea that bacteria often exploit ECM components to establish infections [11, 28, 31].

Plasminogen is a 92 kDa single-chain glycoprotein that undergoes proteolytic cleavage to form double-chained plasmin during the process of fibrinolysis in various animal species [32]. Certain bacteria express specific antigens, which interact with Plg, recruit Plg to obtain proteolytic activity, promote the invasion of pathogenic bacteria, or facilitate their distribution in infected animals [33]. Moreover, some mycoplasma species, including *M. bovis* [9, 14], *M. fermentans* [34], *M. gallisepticum* [35], *M. synoviae* [36, 37], *M. hyopneumoniae* [38], and *M. pneumoniae* [39], have been

shown to bind Plg on their cell surface and activate it to plasmin in the presence of tPA or urokinase plasminogen activator (uPA). Plg interacts with lysine residues on ligands through its kringle domain lysine binding sites. It potentially promotes bacterial adhesion and invasion by connecting Plg-binding proteins on the bacterial surface and host cells [40]. GrpE not only converted plasminogen to plasmin but also played a significant role in enhancing its activation to plasminase via the action of tPA, thereby influencing host fibrinolytic pathways. The inhibition of this activity by ϵ -ACA suggests that GrpE actively participates in modulating fibrinolytic activity in the host system, which may afford *M. bovis* a survival advantage within the infected host. This functional attribute further legitimizes the hypothesis that GrpE is an active participant in the pathogenesis of *M. bovis*. The effects of GrpE on plasminogen activation and binding to ECM components at the bacterial level will be studied in future research.

GrpE elicited significant antibody responses in *M. bovis*-infected and immunized calves but was not reactive to serum from healthy calves. The good immunogenicity of GrpE highlights its potential as a protective antigen, supporting the idea that GrpE may play a critical role in the immune response to *M. bovis*. It is highly conserved and expressed in wild-type strains and other clinical isolates, further reinforcing its potential as a candidate for vaccine development. Recent studies have reported that GrpE is a good vaccine candidate and provides protection against *Mycoplasma synoviae* infection [41]. These findings further underscore the potential of this surface-exposed protein as a novel immunogen for vaccines and therapeutic intervention.

In summary, the chaperone GrpE serves as a multi-functional protein capable of adhering to host cells in *M. bovis*. GrpE interacts with the host ECM and plasminogen, contributing significantly to the pathogenesis of *M. bovis*. GrpE is highly conserved and may be a promising vaccine and drug target for the prevention and control of *M. bovis* infections.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13567-025-01619-4>.

Additional file 1: *M. bovis* strains used in this study. List of *M. bovis* strains used in this study.

Additional file 2: Expression, purification, and identification of rGrpE. **A.** SDS-PAGE analysis of rGrpE purified on a Ni-NTA column. **B** Western blot analysis of GrpE was performed with anti-GrpE serum (1:2000) and anti-6xHis tag monoclonal antibody (1:10 000).

Additional file 3: PCR identification of the grpE gene in the *M. bovis* ΔgrpE strain and its complementation strain. PCR-based identification of the *grpE* gene in the *M. bovis* wild-type strain, *M. bovis* Δ*grpE* strain, and complementation strain *M. bovis* Δ*grpE*:*grpE*.

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Authors' contributions

Shengli Chen conceived, designed, supervised and funded this research. Yuefeng Chusupervised and funded the study. Xiangrui Jin, Shengli Chen and Ahmed Adel Baz wrote the draft manuscript. Xiangrui Jin, Huafang Hao, and Shanyu Jin conducted the experiments. Yifan Zhang, Zhangcheng Li and Shimei Lan contributed reagents and materials. All authors read and approved the final manuscript. => SC conceived, designed, supervised and funded this research. YC supervised and funded the study. XJ, SC and AAB wrote the draft manuscript. XJ, HH, and SJ conducted the experiments. YZ, ZL and SL contributed reagents and materials. All authors read and approved the final manuscript.

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Data availability

The datasets analysed are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The animal tests were performed in accordance with local legislation and institutional requirements and were approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Competing interests

The authors declare that they have no competing interests.

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References

- Dudek K, Szacawa E, Nicholas RAJ (2021) Recent developments in vaccines for bovine mycoplasmoses caused by *Mycoplasma bovis* and *Mycoplasma mycoides* subsp. *mycoides*. *Vaccines* 9:549
- Ambroset C, Peticca A, Tricot A, Tardy F (2022) Genomic features of *Mycoplasma bovis* subtypes currently circulating in France. *BMC Genomics* 23:603
- Pereyre S, Tardy F (2021) Integrating the human and animal sides of *Mycoplasmas* resistance to antimicrobials. *Antibiotics* 10:1216
- Gautier-Bouchardon AV (2018) Antimicrobial resistance in *Mycoplasma* spp. *Microbiol Spectr* 6 <https://doi.org/10.1128/microbiolspec.arba-0030-2018>
- Pryslak T, Menghwar H, Perez-Casal J (2023) Complement-mediated killing of *Mycoplasma bovis* does not play a role in the protection of animals against an experimental challenge. *Vaccine* 41:1743–1752

6. Kaplan BS, Dassanayake RP, Briggs RE, Kanipe CR, Boggiatto PM, Crawford LS, Olsen SC, Menghwar H, Casas E, Tatum FM (2024) An injectable subunit vaccine containing elongation factor Tu and heat shock protein 70 partially protects American bison from *Mycoplasma bovis* infection. *Front Vet Sci* 11:1408861
7. Guo Y, Zhu H, Wang J, Huang J, Khan FA, Zhang J, Guo A, Chen X (2017) TrmFO, a fibronectin-binding adhesin of *Mycoplasma bovis*. *Int J Mol Sci* 18:1732
8. Huang J, Zhu H, Wang J, Guo Y, Zhi Y, Wei H, Li H, Guo A, Liu D, Chen X (2019) Fructose-1,6-bisphosphate aldolase is involved in *Mycoplasma bovis* colonization as a fibronectin-binding adhesin. *Res Vet Sci* 124:70–78
9. Gao X, Bao S, Xing X, Fu X, Zhang Y, Xue H, Wen F, Wei Y (2018) Fructose-1,6-bisphosphate aldolase of *Mycoplasma bovis* is a plasminogen-binding adhesin. *Microb Pathog* 124:230–237
10. Song Z, Li Y, Liu Y, Xin J, Zou X, Sun W (2012) α -Enolase, an adhesion-related factor of *Mycoplasma bovis*. *PLoS One* 7:e38836
11. Adamu JY, Mitiku F, Hartley CA, Sansom FM, Marenda MS, Markham PF, Browning GF, Tivendale KA (2020) *Mycoplasma bovis* mbfN encodes a novel LRR lipoprotein that undergoes proteolytic processing and binds host extracellular matrix components. *J Bacteriol* 203:e00154–20
12. Adamu JY, Wawegama NK, Kanci Condello A, Marenda MS, Markham PF, Browning GF, Tivendale KA (2020) *Mycoplasma bovis* membrane protein MilA is a multifunctional lipase with novel lipid and glycosaminoglycan binding activity. *Infect Immun* 88:e00945–19
13. Zhu X, Dong Y, Baranowski E, Li X, Zhao G, Hao Z, Zhang H, Chen Y, Hu C, Chen H, Citti C, Guo A (2020) Mbov_0503 encodes a novel cytoadhesin that facilitates *Mycoplasma bovis* interaction with tight junctions. *Microorganisms* 8:164
14. Liu S, Li Z, Lan S, Hao H, Jin X, Liang J, Baz AA, Yan X, Gao P, Chen S, Chu Y (2023) LppA is a novel plasminogen receptor of *Mycoplasma bovis* that contributes to adhesion by binding the host extracellular matrix and Annexin A2. *Vet Res* 54:107
15. Zeng B, Wang C, Zhang P, Guo Z, Chen L, Duan K (2020) Heat shock protein DnaJ in *Pseudomonas aeruginosa* affects biofilm formation via pyocyanin production. *Microorganisms* 8:395
16. Tripathi P, Singh LK, Kumari S, Hakiem OR, Batra JK (2020) ClpB is an essential stress regulator of *Mycobacterium tuberculosis* and endows survival advantage to dormant bacilli. *Int J Med Microbiol* 310:151402
17. Bracher A, Verghese J (2015) The nucleotide exchange factors of Hsp70 molecular chaperones. *Front Mol Biosci* 2:10
18. Bracher A, Verghese JC (2023) Nucleotide exchange factors for Hsp70 molecular chaperones: GrpE, Hsp110/Grp170, HspBP1/Sil1, and BAG domain proteins. *Subcell Biochem* 101:1–39
19. Yu F, Dong C, Zhang Y, Che R, Xie C, Liu Y, Zhang Z, Li L, Chen X, Cai X, Wang G, Li Y (2023) GrpE and ComD contribute to the adherence, biofilm formation, and pathogenicity of *Streptococcus suis*. *Arch Microbiol* 205:159
20. Murakami J, Terao Y, Morisaki I, Hamada S, Kawabata S (2012) Group A streptococcus adheres to pharyngeal epithelial cells with salivary proline-rich proteins via GrpE chaperone protein. *J Biol Chem* 287:22266–22275
21. Grudniak AM, Włodkowska J, Wolska KI (2015) Chaperone DnaJ influences the formation of biofilm by *Escherichia coli*. *Pol J Microbiol* 64:279–283
22. Yoneda S, Kawai T, Narisawa N, Tuna EB, Sato N, Tsugane T, Saeki Y, Ochiai K, Senpuku H (2013) Effects of short-chain fatty acids on *Actinomyces naeslundii* biofilm formation. *Mol Oral Microbiol* 28:354–365
23. Scherr TD, Roux CM, Hanke ML, Angle A, Dunman PM, Kielian T (2013) Global transcriptome analysis of *Staphylococcus aureus* biofilms in response to innate immune cells. *Infect Immun* 81:4363–4376
24. Zhang X, Jiang X, Yang L, Fang L, Shen H, Lu X, Fang W (2015) DnaJ of *Streptococcus suis* type 2 contributes to cell adhesion and thermotolerance. *J Microbiol Biotechnol* 25:771–781
25. Cayabyab MJ, Macovei L, Campos-Neto A (2012) Current and novel approaches to vaccine development against tuberculosis. *Front Cell Infect Microbiol* 2:154
26. Aguilo N, Gonzalo-Asensio J, Alvarez-Arguedas S, Marinova D, Gomez AB, Uranga S, Spallek R, Singh M, Audran R, Spertini F, Martin C (2017) Reactogenicity to major tuberculosis antigens absent in BCG is linked to improved protection against *Mycobacterium tuberculosis*. *Nat Commun* 8:16085
27. Tang Y, Guo F, Lei A, Xiang J, Liu P, Ten W, Dai G, Li R (2020) GrpE immunization protects against *Ureaplasma urealyticum* infection in BALB/C mice. *Front Immunol* 11:1495
28. Lan S, Li Z, Hao H, Liu S, Huang Z, Bai Y, Li Y, Yan X, Gao P, Chen S, Chu Y (2023) A genome-wide transposon mutagenesis screening identifies LppB as a key factor associated with *Mycoplasma bovis* colonization and invasion into host cells. *FASEB J* 37:e23176
29. Chen X, Huang J, Zhu H, Guo Y, Khan FA, Menghwar H, Zhao G, Guo A (2018) P27 (MBOV_RS03440) is a novel fibronectin binding adhesin of *Mycoplasma bovis*. *Int J Med Microbiol* 308:848–857
30. Vaca DJ, Thibau A, Schütz M, Kraiczky P, Happonen L, Malmström J, Kempf VAJ (2020) Interaction with the host: the role of fibronectin and extracellular matrix proteins in the adhesion of gram-negative bacteria. *Med Microbiol Immunol* 209:277–299
31. Wang J, Li Y, Pan L, Li J, Yu Y, Liu B, Zubair M, Wei Y, Pillay B, Olaniran AO, Chiliza TE, Shao G, Feng Z, Xiong Q (2021) 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* 52:80
32. Xu QY, Pan Q, Wu Q, Xin JQ (2022) *Mycoplasma bovis* adhesins and their target proteins. *Front Immunol* 13:1016641
33. Jeffery C (2018) Intracellular proteins moonlighting as bacterial adhesion factors. *AIMS Microbiol* 4:362–376
34. Yavlovich A, Rechnitzer H, Rottem S (2007) Alpha-enolase resides on the cell surface of *Mycoplasma fermentans* and binds plasminogen. *Infect Immun* 75:5716–5719
35. Fürnkranz U, Siebert-Gulle K, Rosengarten R, Szostak MP (2013) Factors influencing the cell adhesion and invasion capacity of *Mycoplasma gal-lisepticum*. *Acta Vet Scand* 55:63
36. Bao S, Guo X, Yu S, Ding J, Tan L, Zhang F, Sun Y, Qiu X, Chen G, Ding C (2014) *Mycoplasma synoviae* enolase is a plasminogen/fibronectin binding protein. *BMC Vet Res* 10:223
37. Qi J, Wang Y, Li H, Shang Y, Gao S, Ding C, Liu X, Wang S, Li T, Tian M, Yu S (2022) *Mycoplasma synoviae* dihydrolipoamide dehydrogenase is an immunogenic fibronectin/plasminogen binding protein and a putative adhesin. *Vet Microbiol* 265:109328
38. Wang J, Li S, Chen J, Gan L, Wang J, Xiong Q, Feng Z, Li Q, Deng Z, Yuan X, Yu Y (2023) Hijacking of host plasminogen by *Mesomycoplasma (Mycoplasma) hyopneumoniae* via GAPDH: an important virulence mechanism to promote adhesion and extracellular matrix degradation. *Microbiol Spectr* 11:e0021823
39. Thomas C, Jacobs E, Dumke R (2013) Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*. *Microbiology* 159:352–365
40. Raymond BB, Djordjevic S (2015) Exploitation of plasmin(ogen) by bacterial pathogens of veterinary significance. *Vet Microbiol* 178:1–13
41. Zhang G, Han L, Li Z, Chen Y, Li Q, Wang S, Shi H (2023) Screening of immunogenic proteins and evaluation of vaccine candidates against *Mycoplasma synoviae*. *NPJ Vaccines* 8:121

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