\( \alpha \)-Enolase, an Adhesion-Related Factor of *Mycoplasma bovis*

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**Abstract**

*Mycoplasma bovis* is the causative agent of *Mycoplasma bovis*-associated disease (MbAD). Although the mechanisms underlying *M. bovis* adherence to host cells is not clear, recent studies have shown that the cell surface protein \( \alpha \)-enolase facilitates bacterial invasion and dissemination in the infected host. In this study, we cloned, expressed and purified recombinant *M. bovis* \( \alpha \)-enolase and induced polyclonal anti-\( \alpha \)-enolase antibodies in rabbits. *M. bovis* \( \alpha \)-enolase was detected in the cytoplasmic and membrane protein fractions by these antibodies. Triple immunofluorescence labeling combined with confocal laser scanning microscopy (CLSM) revealed that the plasminogen (Plg) enhanced the adherence of *M. bovis* to embryonic bovine lung (EBL) cells; the values obtained for adherence and inhibition are consistent with this finding. Interestingly, we found that trace amounts of trypsin acted as a more effective enhancer of cell adherence than Plg. Hence, our data indicate that surface-associated *M. bovis* \( \alpha \)-enolase is an adhesion-related factor of *M. bovis* that contributes to adherence by binding Plg.

**Introduction**

The adherence of mycoplasmas to the host cell initiates infection with bacteria of this genus [1]. *Mycoplasma bovis* is the causative agent of *Mycoplasma bovis*-associated disease (MbAD) [2]. The bacterium was first isolated from a case of severe mastitis by Hale in 1961 [3]. *M. bovis*, which causes pneumonia, otitis media and arthritis in young calves, has been an important cause of disease in North America, Europe and Asia [4–6]. *M. bovis* was first isolated in the Hubei province of China in 2008 [6], but the economic cost of MbAD has not been reported.

Plasminogen (Plg) is a single-chain glycoprotein (with a molecular mass of 92 kDa) that is converted into plasmin [7]. Many bacteria express surface structures that interact with Plg and specific receptors on their cell surfaces enhance the activation of Plg via Plg activators [8]. Many pathogens have been found to capture Plg, which allows the bacteria to acquire surface-associated proteolytic activity that may facilitate bacterial invasion and dissemination in the infected host [9].

Recently, the glycolytic enzyme \( \alpha \)-enolase, a non-classical Plg-binding protein, has been found in many bacteria [9–12]. Prokaryotic \( \alpha \)-enolase is a highly conserved protein that may contribute to pathophysiological processes [13]. Research has shown that the presence of bacterial surface \( \alpha \)-enolase is closely related to bacterial adherence to the host cell [14]. There is, however, no evidence showing that *M. bovis* \( \alpha \)-enolase (MbEno) is a membrane protein related to *M. bovis* adherence to the host cell.

In this study, we found that *M. bovis* expresses several plasminogen-binding proteins. We used recombinant *M. bovis* \( \alpha \)-enolase (rMbEno) to induce anti-\( \alpha \)-enolase antibodies in rabbits to facilitate characterization of the adherence properties of *M. bovis* to embryonic bovine lung (EBL) cells. We also explored the role of \( \alpha \)-enolase as a Plg-binding protein in adherence and invasion of *M. bovis*.

**Results**

Identification of enolase of *M. bovis*

The 1365-bp open reading frame (ORF) of \( \alpha \)-enolase was identified in the complete genomic sequence of *M. bovis* strain Hubei. The ORF encoded a 454-amino-acid protein with a theoretical molecular weight of 49369 Da and isoelectric point of 5.27 (Pepstats V6.0.1). The *M. bovis* \( \alpha \)-enolase lacks classical protein-sorting signals such as N-terminal signal peptides, hydrophobic domains, or a C-terminal LPXTGX motif (SOSUI). The amino acid sequence was homologous to the \( \alpha \)-enolase sequences from a variety of species, as determined using a maximum-likelihood analysis in MEGA4.0.2. The *M. bovis* Hubei \( \alpha \)-enolase identified showed more than 90% homology to *M. bovis* PG45 (E4PX0), *M. fermentans* (E1PS24), *M. agalactiae* (A5IYA8), *M. hyopneumoniae* (Q601S2) and *M. gallisepticum* (Q2NAY0), respectively. In addition, the protein contained features typical of Plg-binding-site motifs including lysine as the C-terminal residue.
(FYNIK), and a conserved, positively charged lysine-rich internal motif [LYDENSKKY], as identified by UniProt (data not shown).

**M. bovis α-enolase gene expression, and protein purification**

We designed primers to mutate TGA into TGG to obtain a sequence that would be correctly expressed in E. coli. The recombinant plasmid was digested with restriction enzymes to verify the size of the insert DNA and that TGA (186 bp and 960 bp) had been successfully mutated into TGG.

Recombinant plasmids were transformed into E. coli BL21 (DE3) pLysS cells to obtain the recombinant fusion protein designated His-rMbEno. His-tagged recombinant protein, purified under non-denaturing conditions (using Ni-NTA His Bind Resin) had an apparent molecular weight of 72 kDa.

**The α-enolase antibody**

Ten days after the third immunization, the reactivity and specificity of the rabbit antisera was tested by enzyme-linked immunosorbent assay (ELISA) (Figure 1). Following purification with Protein A sepharose, the serum, containing anti-rMbEno polyclonal antibodies (2.0 mg/ml), was stored at −20°C.

**Localization of M. bovis α-enolase**

MbEno was detected in the cell-soluble cytosolic fraction proteins (Figure 2, lane 2), in the cell-membrane-fraction proteins (Figure 2, lane 3) and in whole-cell proteins (Figure 2, lane 4). Bovine serum albumin (Figure 2, lane 1) and rMbEno (Figure 2, lane 5) were employed as negative and positive controls, respectively. This analysis, using anti-rMbEno antibodies, revealed a strong reactivity to a protein of approximately 49 kDa, suggesting that MbEno is present in both the membrane and the soluble cytosolic protein fractions of M. bovis cells.

**M. bovis and rMbEno bind plasminogen**

MbEno was detected among the cell-membrane-fraction proteins (Figure 3, lane 1) and cell-soluble cytosolic-fraction proteins (Figure 3, lane 2), in the cell-membrane-fraction proteins (Figure 3, lane 3), recombinant M. bovis α-enolase (lane 4), and purified recombinant M. bovis α-enolase blotted onto a nylon membrane and detected with rabbit anti-recombinant enolase antibodies (lane 5) and BSA (lane 5) blotted onto a nylon membrane and detected with rabbit anti-recombinant enolase antibodies. M: protein marker.

**Adherence and inhibition assay**

M. bovis has been shown to be capable of adhering to EBL cells, and it has been further shown that membrane-protein antibodies can significantly inhibit adherence of the bacterium to such cells.
Figure 5 shows the M. bovis Hubei strain adhering to EBL cells pretreated with Plg. We found that adherence to the cells was inhibited by treatment with the anti-rMbEno antibody. In a competition assay, however, we found that infection was not inhibited by treatment with non-immune rabbit antibodies. Furthermore, no fluorescence was detected when EBL cells were incubated with the secondary antibody alone. The results of the confocal laser scanning microscopy (CLSM), therefore, show that α-Enolase is an M. bovis adhesion-related factor.

To further validate these experiments, adherence and inhibition values were calculated. Table 1 shows that after pre-incubation with Plg, M. bovis EBL cell adherence rates were enhanced by 11.9%. We also noted that proteolysis of the Hubei strain by trypsin resulted in enhanced adherence to EBL cells; this was more effective than that observed with Plg alone.

We observed no obvious difference between the anti-rMbEno antibodies and non-immune rabbit serum in binding inhibition activity tests (P>0.05), because EBL cells were not treated with Plg. The inhibition values also showed no obvious differences between M. bovis pretreated with trypsin by anti-rMbEno antibody and the non-immune rabbit serum (P>0.05). However, when the EBL cells were treated with Plg, the anti-enolase antibodies (that inhibited adherence) were more effective than the non-immune rabbit antibodies at inhibiting adherence (P<0.05).

Discussion

Identification of the complete genomic sequence of M. bovis (Hubei strain) α-enolase enabled us to study the α-enolase of this organism. The M. bovis α-enolase sequence contains the typical Plg-binding-site motifs that characterize α-enolase proteins. However, M. bovis α-enolase lacks classical protein-sorting signals. If the α-enolase is not a surface protein, the function of α-enolase as a Plg-binding protein in M. bovis may have less biological significance. However, α-enolase has been shown to be a surface protein in many bacteria species [11,13,14]. How α-enolase molecules lacking signal peptides are exported to the cell surface is an unresolved question. Secretion of α-enolase has been found to be SecA2-dependent in Listeria monocytogenes [15]; therefore, some researchers have suggested that a similar mechanism may be used to export α-enolase [14]. However, if such a mechanism exists, this presents an important and challenging task that needs to be addressed by further experimentation. Our experiments suggest that M. bovis membrane-associated α-enolase is a Plg-binding protein.

In this study, we have provided evidence that the α-enolase is present in both the soluble cytosolic fraction and the membrane fraction of M. bovis; a similar finding has been made in M. fermentans using transmission electron microscopy [10]. Figure 4 (lanes 2 and 3) show the presence of MbEono in both the membrane and soluble cytosolic protein fractions of M. bovis. This analysis demonstrates that α-enolase is a membrane-associated.
protein in *M. bovis*, as has been found in many other bacterial species [9–12]. We suggest that the presence of α-enolase in this location is consistent with the etiology of adherence to host cells.

That α-enolase plays a role in the invasion of host tissue by pathogens exhibiting adhesion has already been demonstrated [13]. Recent studies indicated that α-enolase on the cell surface of *Streptococcus pneumoniae* and *M. fermentans* binds and activates plasminogen [10,16] and influences *Streptococcus pneumoniae* adherence to human pharyngeal cells [16]. α-enolase confers surface-associated proteolytic activity, which may facilitate pathogen invasion and dissemination in the infected host [9].

Interestingly, western immunoblot assays revealed that several different proteins within the *M. bovis* membrane protein fraction bound to Plg. Figure 4 shows that the rabbit anti-rMbEno antibody inhibits the binding of Plg to *M. bovis* whole-cell proteins, supporting our hypothesis that α-enolase is a major Plg-binding protein in *M. bovis*.

Our research supports the hypothesis that the *M. bovis* α-enolase is a Plg-binding protein, and we speculate that α-enolase is involved in *M. bovis* adhesion to EBL cells. The triple immunofluorescence labeling technique used here, when combined with CLSM, offers a visual way to differentiate between adherence and adherence inhibition in mycoplasmas. Utilization of CLSM revealed that the degree of *M. bovis* adherence to the cell surface of EBL cells is related to the availability of Plg and α-enolase.

The adherence and inhibition assay results confirm that Plg facilitates the binding of *M. bovis* to EBL cells. Surprisingly, proteolysis of *M. bovis* by trypsin resulted in significantly enhanced binding to EBL cells in comparison with Plg alone, and the enhancement was not specifically inhibited by the addition of anti-

### Table 1. Adherence and inhibition assays.

| antibodies                | Pretreatments          | HBSS (%) | Plasminogen (0.25 unit/ml) (%) | Trypsin (25 µg/ml) (%) |
|---------------------------|------------------------|----------|-------------------------------|-----------------------|
| Negative control          | 100                    | 111.9    | 234.9                         |
| Non-immune rabbit antibody| 59.4                   | 69.9*    | 39.7                          |
| Rabbit Anti-enolase antibody | 57.0                  | 58.8*    | 37.6                          |

Experiments were performed in triplicate. Hank’s Balanced Salt Solution (HBSS).

*P* < 0.05, compared with the corresponding group using non-immune rabbit antibodies.

doi:10.1371/journal.pone.0038836.t001

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![Figure 5. Confocal laser scanning microscopy depicting interactions between the Mycoplasma bovis Hubei strain and embryonic bovine lung (EBL) cells.](image)}
rMbeno antibody. This result suggests that other protease-binding regions in the M. bovis cell membrane are involved in proteolysis, as has been described previously [17], and that these regions enhance Mycoplasma adherence to host cells. Some research has shown that proteases such as trypsin and proteinase K reduced mycoplasma binding to cells by hydrolyzing the surface adhesion-related protein [18]. We believe that the presence of large numbers of proteases would reduce binding by hydrolysis of surface proteins; however, in the presence of trace amounts of protease, the protease would enhance binding by giving mycoplasmas proteolytic activity. Essentially, whether protease pre-treatment makes mycoplasmas more effective at binding cells is probably dependent on the concentration of the protease itself and the reaction conditions present at the time.

α-enolase has been detected in studies attempting to identify proteins interacting with host surfaces in bacteria [12,16]. To functionally characterize the M. bovis α-enolase, both the recombinant and the native proteins were subjected to an in vitro plasminogen-binding assay. In this work we have demonstrated the ability of M. bovis α-enolase to bind Plg; this could be of great importance for Mycoplasma establishment in the host, allowing adhesion to the EBL cells. Other studies have shown that α-enolase is not only an adhesion-related factor, but also an autoantigen in connective tissue diseases [19]. We speculate that α-enolase may be involved in various clinical and pathologic sequelae of M. bovis infection, such as arthritis, tenosynovitis and meningitis.

Conclusions
In conclusion, our studies show that the α-enolase of M. bovis is a surface-exposed protein that enables M. bovis adherence to EBL cells by binding Plg. However, several other Plg receptors exist on the M. bovis cell surface. Our finding that trypsin enhances adherence more effectively than does Plg is noteworthy and may provide some insight towards identifying the mechanisms underlying M. bovis adherence to host cells.

Materials and Methods
Ethics Statement
Animal experiments were approved by Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences and National Engineering Research Center of Veterinary Biologics. And animal experiments were performed in accordance with animal ethics guidelines and approved protocols. The Animal Ethics Committee approval number was Heilongjiang-SYXK 2006-032.

Bacterial strains, cell lines, and culture conditions
The M. bovis strain Hubei isolated in China [6], was used in this study. In these experiments, mycoplasmas were grown in modified pleuropneumonia-like organism (PPLO) medium containing 20% donor equine serum (HyClone, Logan, USA), 10% yeast extract, thallium acetate (0.125 mg/ml) and penicillin (200 IU/ml) [20].

The EBL cells [21] were kindly provided by Pro. Xue (National Engineering Research Center of Veterinary Biologics, Harbin, China).

Cloning, M. bovis α-enolase gene expression, and protein purification
The α-enolase ORF was identified from the M. bovis strain Hubei strain complete genome sequence [22]. The M. bovis α-enolase ORF containing two TGA codons, which were in vitro terminators, encoded tryptophan in Mycoplasma. Primers eno1/eno2, eno3/eno4 and eno5/eno6 (Table 2) were designed for site-directed mutagenesis and used in overlapping PCRs. The full-length mutated gene was expressed by pET System (Novagen, Madison, USA). The resulting rMbEno protein was purified using HisPur Ni-NTA Resin and Kits (Thermo, Rockford, USA).

Antibody production
Polyclonal antibodies against M. bovis α-enolase were prepared in female New Zealand White rabbits by injection with rMbEno protein. The rabbits were bled ten days after the third immunization and the antibody titers measured by ELISA. Antibodies were purified using Protein A High-Capacity Agarose and Kits (Thermo) and quantified using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Nan Tong, China).

Preparation of M. bovis protein fractions
Membrane and cytosolic protein fractions from M. bovis were obtained using ProteoExtract Transmembrane Protein Extraction Kit (Novagen) according to the manufacturer’s instructions. Whole cell protein extracts were prepared by sonication. Protein quantitation was performed with the BCA Protein Assay Kit.

Cellular localization of M. bovis α-enolase
To locate the cellular distribution of MbEno, Western immunoblot assays were performed as described previously [14]. Briefly, the gels were transferred to nitrocellulose membranes (PALL, Ann Arbor, USA) and blocked with 5% gelatin derived from cold-water fish skin (Sigma). Following three washes with PBST (PBS containing 0.05% Tween-20), nitrocellulose(NC) membrane was incubated with anti-rMbEno antibody (1:800). Bound antibodies were detected by incubation with anti-rabbit IgG (whole-molecule) peroxidase conjugate (Sigma). Cross reacting protein bands were visualized using a DAB Substrate Kit (Thermo).

M. bovis α-enolase plasminogen binding assays
A ligand blotting assay was performed to test the ability of the M. bovis α-enolase to bind plasminogen, as described previously.

| Primers name | Sequence (5’→3’) | Localization (nt) |
|--------------|-----------------|------------------|
| Eno1         | cag gga tcc atc ctt att att gaa acc att caa g tca taa | 1–186 |
| Eno2         | cac cttgc cga acc agt ttc ctt cggc cgc aaa ggt | 186–960 |
| Eno3         | gtt atg acc gcc gtt gat att a | 960–1365 |
| Eno4         | tgc ctt tca gga aga cca tct tca att gaa ata ata gga t tct | |
| Eno5         | tgc tga aag cga tgg gga agg att tgc aaa aat gac t cgg | |
| Eno6         | gtc gac ctt ctt tat gtt gta aaa tgc ttt aga acc | |

doi:10.1371/journal.pone.0038836.t002
Briefly, *M. bovis* fractionated proteins and rMbEno were transferred onto NC membranes, and incubated with P1g (Sigma). Blots were developed with DAB Substrate Kit.

ELISA was performed to verify the ability of the proteins to bind P1g [14]. ELISA plate wells were coated with rMbEno. In a parallel experiment, ELISA wells were coated with membrane protein fraction from *M. bovis* cells and a range of concentrations of P1g added to the wells. The inhibition experiment was performed by adding anti-rMbEno antibody (in serial dilutions from 1/100 to 1/6,400) prior to the addition of P1g [14]. The inhibition experiment was performed by adding anti-rMbEno antibody in serial dilutions from 1/100 to 1/6,400 prior to the addition of P1g. A set of competition experiments were also performed that included the addition of anti-*M. bovis* monoclonal antibody (Abcam, Hong Kong, China). Protein-protein interactions were identified through use of sheep anti-plasminogen polyclonal antibodies. The binding was determined by incubation with anti-sheep antibodies. The absorbance was measured at A450 using a microplate reader (Bio-Tek Instruments, Winooski, USA).

Adherence and inhibition assay of *M. bovis*

Invasion of *M. bovis* strain Huibeii was determined using CLSM [23]. After washing to remove non-adherent mycoplasmas, *M. bovis*-infected EBL cells were fixed with 4% paraformaldehyde (PFA). The dish containing the cells was overlaid with anti-*M. bovis* antisera. Antibodies bound to the cells were detected by rabbit anti-bovine IgG (whole molecule)-FITC (Sigma). The membranes containing EBL cells were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). Cell nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescent was evaluated using a Leica TCS SP5 laser scanning confocal microscope (Leica, Mannheim, Germany).

Adherence and inhibition of adherence was determined using a bacteriological assay, as described previously [24]. P1g(0.25 units/ml) was incubated with EBL cells. The cells then were infected with *M. bovis* (Hubeii) at a multiplicity of infection (MOI) of 200. Non-adherent mycoplasmas were removed by washing. The cells were lysed with 0.25% trypsin (Gibco) and serial dilutions of the cell lysate were plated onto solid modified PPLO medium. The *Mycoplasma* colonies were counted to determine the adherence frequencies. For the adherence inhibition assay, *M. bovis* (Hubeii) was incubated with rabbit antibody raised against rMbEno (20 μg/ml), or non-immune rabbit antibody (20 μg/ml) prior to infecting the EBL cells (as described above). A parallel experiment was performed by replacing the native *M. bovis* preparation in the adherence reaction mixture with *M. bovis* that had been pre-incubated with trypsin (25 μg/ml) for 15 min at 37°C (all steps thereafter were performed as described above). The percent inhibition was calculated using the following formula: (CFU each cell treated with rabbit antibody/CFU each untreated cell) × 100.

Acknowledgments

We thank Dr. Tao from Harbin Veterinary Research Institute for excellent technical assistance with the CLSM experiments.

Author Contributions

Conceived and designed the experiments: ZS JX. Performed the experiments: JX Y. Li. Analyzed the data: ZS Y. Li. Contributed reagents/materials/analysis tools: Y. Liu XZ. Wrote the paper: ZS WS.

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