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Mycoplasma Bovis adhesins and their target proteins

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Bovine mycoplasmosis is an important infectious disease of cattle caused by *Mycoplasma bovis* (*M. bovis*) which poses a serious threat to the breeding industry. Adhesin is involved in the initial process of *M. bovis* colonization, which is closely related to the infection, cell invasion, immune escape and virulence of this pathogenic microorganism. For the reason that *M. bovis* lacks a cell wall, its adhesin is predominantly located on the surface of the cell membrane. The adhesins of *M. bovis* are usually identified by adhesion and adhesion inhibition analysis, and more than 10 adhesins have been identified so far. These adhesins primarily bind to plasminogen, fibronectin, heparin and amyloid precursor-like protein-2 of host cells. This review aims to concisely summarize the current knowledge regarding the adhesins of *M. bovis* and their target proteins of the host cell. Additionally, the biological characteristics of the adhesin will be briefly analyzed.

KEYWORDS

M. bovis, adhesin, adhesin binding protein, bovine, vaccine development

Introduction

M. bovis, the causal agent of bovine mycoplasmosis, is a cell wall-free microorganism belonging to the genus *Mycoplasma* and family *Mycoplasmataceae* within the class *Mollicutes*. Bovine mycoplasmosis, currently prevalent all over the world, is one of the severe bovine diseases that cause immeasurable losses to the cattle industry (1). The clinical signs of bovine mycoplasmosis can be manifested as mastitis, pneumonia, arthritis, skin abscess, meningitis, otitis and reproductive tract infection. Furthermore, *M. bovis* is also one of the main pathogens of the bovine respiratory disease complex (BRDC) (2, 3). Cattle are the most vulnerable animal to *M. bovis*, all age groups (preweaning, postweaning, neonate and adult) and all cattle sectors such as beef, milk or rearing could be affected (1). *M. bovis* often causes mixed infection with *Pasteurella multocida* (4), *Haemophilus somnus* (5), bovine respiratory syncytial virus (BRSV) (6), bovine herpesvirus 1 (BHV-1) (7), bovine viral diarrhoea virus (BVDV) (5), *Histophilus somni* (8) and parainfluenza virus type 3 (6). There is synergy between *M. bovis* and these pathogens during mixed infection.

M. bovis was first isolated from a dairy farm with severe mastitis cows in the United States in 1961 (9). Although it has been over 60 years since *M. bovis* was first isolated, the pathogenesis of *M. bovis* is still not very clear. The lack of genomic manipulation methods and expensive animal models cause diagnosis, treatment and vaccine research to stall. It has been confirmed that *M. bovis* infection has consequences on the host immune system, including the production of inflammatory factors or apoptosis of immune cells, and membrane proteins play a critical role in regulating the host defense system (10, 11). In addition, some metabolites of *M. bovis* can also induce host inflammatory reactions, especially following *M. bovis* colonization, it will produce hydrogen peroxide, cause host cell damage in parasitic sites, and mediate more serious inflammatory reactions.

Adhesion of *M. bovis* to host cells

There is a perception that the cell membrane contact is more conducive to the fusion of Mycoplasma-host cell membrane and the exchange of intracellular components (10). Adhesion is an important step in *M. bovis* infection, the functional proteins (known as adhesins) are closely linked to the pathophysiological process (12, 13). Traditionally, it is generally believed that *M. bovis* mainly parasitizes outside cells, but mounting evidence shows that *M. bovis* can invade host cells, and this mechanism is related to its distribution in host animals and immune escape (14–16). To date, *M. bovis* has been detected in neutrophils, macrophages, bronchial epithelial cells, hepatocytes and kidney cells of infected cattle (17, 18). *In vitro* experimental results also showed that *M. bovis* can invade T cells, B cells, dendritic cells and peripheral blood mononuclear cells such as monocytes and erythrocytes (16). The target proteins of bacterial adhesins are frequently involved in the process of pathogen entry into cells, such as the binding of *Staphylococcus aureus* receptors to fibronectin (FN), which in turn recruits integrins to invade target cells through the endocytic pathway (19). *M. pneumoniae* associates with integrin $\beta 1$ on the surface of epithelial cells *via* interactions with surface-bound fibronectin and initiates signaling events that stimulate pathogen uptake into clathrin-coated vesicles and caveosomes (20). Some adhesins of *M. bovis* also take FN as the target protein, but whether *M. bovis* invades host cell also adopts the this mechanism remains to be further studied, although recent research shows that clathrin-dependent endocytosis is one of the major pathways by which *M. bovis* invades into synovial cells (14). The invasion of *M. bovis* into different cells may contribute to the spread of pathogens to various colonization sites, which is related to weakening the therapeutic effect of antibiotics and escaping from the immune system killing. *M. bovis* primarily infects cattle, but sheep, goats, pigs, deer and human beings have also been reported to be infected, and *M. bovis* has been isolated

from the respiratory tract, udder, joint, heart, brain and other parts of sick cattle (11). The phenomenon of *M. bovis* infecting a variety of animals, tissues and cells suggests that the adhesins of *M. bovis* may be diverse and complex. So far, there are great room for improvement of *M. bovis* vaccine products and the detection methods of *M. bovis*. Furthermore, *M. bovis* is very prone to drug resistance (21), all of these reasons make the prevention, control and elimination of the disease very difficult. Theoretically, the adhesins of mycoplasma are expected to be the candidate vaccine component. In addition, many adhesins have the potential to establish serological detection methods for their excellent immunogenicity.

Adhesin binding protein

The adhesion of pathogens to specific tissues or cells, a complex process, is the beginning of infection. The target proteins of bacterial adhesins are mainly the components of the host extracellular matrix (ECM), and the protein components of ECM include collagen, elastin, FN, platelet-derived growth factors, laminin and so on (22). For mycoplasma, proteins located on the cell membrane can also bind to ECM components of host cells, such as collagen, laminin, FN, plasminogen (Plg) and glycosaminoglycan heparin, thus mediating mycoplasma colonization or invasion (23). Currently, there are four types of *M. bovis* adhesin binding proteins that have been identified, namely FN, Plg, heparin and amyloid precursor-like protein-2 (APLP-2).

FN is a multifunctional glycoprotein with a high molecular weight, which exists in the forms of soluble dimer in body fluid and insoluble dimer outside cells. The heterodimer is the main form of FN, which is composed of two 230–270 kDa protein chains (24). The dimers are connected by disulfide bonds at the C-end, and each chain is composed of three different types of repeated protein units. These repeating units are called FN domains, which are the basis for FN to perform various functions (25–27). The FN is very conservative in higher animal species. Its key function is to connect the cytoskeleton with the exocellular matrix. FN is one of the crucial binding proteins of bacterial adhesin. At present, more than 100 kinds of bacterial FN target proteins have been identified (25). There is growing evidence that FN and its hydrolysates are related to signal pathways, implying that FN may have other important significance for bacterial infection and invasion in addition to being the target protein of adhesin (27).

Plg is a single chain glycoprotein with a molecular weight of approximately 92 kDa (28). The surface structure of many bacterial pathogens can interact with Plg. These pathogenic microorganisms will recruit Plg to obtain proteolytic activity, promote the invasion of pathogenic bacteria or facilitate their distribution in infected animals (29, 30). Mature Plg mainly exists in two forms: Glu-Plg and Lys-Plg. Glu-Plg is converted

into Lys-Plg if the 77 amino-terminal peptides are removed. Both forms of Plg contain seven domains: one activating peptide located in the N-terminal region known as the PAP domain (1 – 77 aa), five kringle domains (Kr1 – 5) and one SP serine protease domain (562 – 791 aa). Plg is an adhesion receptor of a variety of bacteria, fungi and parasites, which is related to the pathogenesis and immune escape of these pathogens (31).

Heparan sulfate (HS) is a widespread form of sulfated glycosaminoglycans, which is present in all types of tissues and cells at extracellular and cellular levels. HS consists of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and hexuronic acid residues. Structurally, the multiple binding activities of HS are closely linked to its extended structural variability. The HS chains are synthesized in the Golgi apparatus by enzymes that initially polymerize alternating N- GlcNAc and glucuronic acid residues. The resulting disaccharide repeats will be variously modified by interdependent reactions, and these modification reactions will not occur uniformly along the chain (32). This mechanism will cause the HS chain to have a wide range of sulfation modes and differential isomerization characteristics, resulting in great diversity, and then provide HS chains with different docking sites for various ligands of polysaccharides. It has been proved that HS proteoglycan is a target protein for the adhesion of a variety of pathogens, which may participate in the internalization process of pathogens and is closely related to the pathogenic mechanism (33). Such as lysated products of *M. hyopneumoniae* P159 binding to heparin facilitated colonized on PK15 cell and may be related to this pathogen internalization (34), the binding of *Candida albicans* to heparin is related to biofilm formation (35), SARS-CoV-2 also applied heparan sulfate as receptor, and several sulfated polysaccharides including heparin show potent anti-SARS-CoV-2 activity (36).

Amyloid precursor-like protein 2 (APLP2) is a member of the amyloid precursor protein family of proteins (APP). APP was found to be evolutionary highly conserved. All APP family members are type 1 integral membrane proteins with a single membrane-spanning domain, a large ectoplasmic N-terminal region and a shorter cytoplasmic C-terminal region (37). The sequences of all APP homologues can be divided into similar domain structures as APP. The ectoplasmic region of APP, which constitutes the major part of the protein, can be divided into the E1 and E2 domains (38). The E1 domain can be further divided into a number of subdomains, including a heparin-binding/growth-factor-like domain (HFBD/GFLD), a copper-binding domain (CuBD) and a zinc-binding domain (ZnBD). The E2 region consists of another HFBD/GFLD and a random coil (RC) region (37). The biological function of APP is still not fully clear. However, it is known that the APP family proteins have redundant and partly overlapping functions (37). The discrete functions of APP including cell adhesion, dendritic outgrowth, axonal transport, synapse formation and synapse modulation, and this diversity of roles is attributed to its

numerous proteolytic products (39). Although APP and APLP-2 are related genetically, it has been observed that they are transcriptionally divergent, and there are unique sequence motifs in each gene that suggest specialized, non-overlapping functions (40). APLP-2 has been documented as contributing to pancreatic cancer cell migration, invasiveness, metastasis and copper homeostasis (41). In addition, APLP-2 is an essential component for cell adhesion. There are few reports on APLP-2 as a pathogen receptor, but it has been confirmed that APLP-2 is a target protein for the adhesion of *M. bovis* (42).

Adhesion of *M. bovis*

So far, 16 proteins have been identified to be involved in the adhesion of *M. bovis* (Table 1). These adhesins are basically located on the cell surface. Among these adhesins, seven adhesins have been identified with the clear target protein, three adhesion-related proteins can interact with two different host proteins, and four adhesins are moonlighting proteins with enzyme activities in addition to binding function.

NADH oxidase

NADH oxidase (NOX) is encoded by the NOX gene, and this protein contains 454 amino acids with a molecular weight of 49 kDa (42). NOX has no signal peptide or transmembrane region. The characterization of prokaryotic recombinant NOX protein suggested that it not only had the catalytic activity of oxidase but also functioned as an adhesin. The NOX protein was shown to be distributed in the cytoplasm and cell membrane of *M. bovis*, but it could not be secreted into the culture supernatant. The recombinant NOX protein can bind to EBL cells. Further study found that the protein binds to the membrane protein and cytoplasmic protein of EBL cells in a dose-dependent manner. The reaction with membrane protein is stronger than cytoplasmic protein, and this binding can be specifically blocked by anti-NOX serum. Furthermore, the NOX protein and anti-NOX serum can also block the adhesion of *M. bovis* to EBL cells in a dose-dependent manner. Compared to the parent, a NOX protein-deficient strain showed a decrease in adhesion and H₂O₂ production. Subsequent experiments confirmed that NOX could specifically adhere to amyloid precursor protein 2 (APLP-2) and FN.

leucine-rich repeat lipoprotein

James and colleagues found that a leucine-rich repetitive lipoprotein (LRR) has adhesin activity (43). This protein is encoded by the *mbfn* gene of *M. bovis* standard strain PG45. The western blot analysis confirmed that a 48 kDa protein exists

TABLE 1 Summary of *M. bovis* adhesin information.

Adhesin	NCBI Protein ID	No. of aa	MW (kDa)	Signal peptide	Transmembrane region	Target protein	Antigenicity	Subcellular Localization	Moonlight function	Reference
NOX	WP_013954704.1	454	49kDa	No	No	APLP-2 and FN	Unknown	Cytoplasm and cell membrane	Yes	42
MbfN	ADR25360.1	612	96 kDa	Sec/SPII (20-21)	No	FN and heparin	Yes	cell surface	Unknown	43
FBA	WP_013954544.1	291	34kDa	No	No	FN and Plg	Yes	Cytoplasm and cell membrane	Yes	44/45
TrmFO	AEI89864.1	427	8.548kDa	No	No	FN	Yes	Cytoplasm and cell membrane	Unknown	52
α -Enolase	AEI90157.1	455	49.369kDa	No	No	Plg	Yes	cell surface	Yes	55
MilA	ADR24994.1	2670	303kDa	Sec/SPI (38-39)	7-29	Haperin	Yes	Whole cell and culture supernatant	Yes	57
P27	MBOV_RS03440	241	27.1kDa	Sec/SPII (24-25)	No	FN	Yes	Cytoplasm and cell membrane	Unknown	58
VpmaX	AEI90145.1	229	35kDa	Sec/SPII (25-26)	7-29	Unknown	Unknown	Cell membrane and whole-cell	Unknown	61
P26 protein	Unknown	Unknown	32kDa	Unkonwn	Unknown	Unknown	Yes	Unknown	Unknown	64
VSPs*	Unknown	Unknown	Unknown	Sec/SPII (23-24/24-25)	No	Unknown	Yes	Cell membrane	Unknown	13/67
Mbov-0503	AFM51859.1	548	59.48kDa	No	7-29	Unknown	Unknown	Cell membrane	Unknown	68
24kDa protein	Unknown	Unknown	24kDa	Unkonwn	Unknown	Unknown	Unknown	Unknown	Unknown	69

*VSPs including VspA, VspB, VspC, VspE and VspF.

in the eight *M. bovis* strains used in their research. In addition to the 48 kDa protein, some strains also have a 70 kDa band. The difference between the two proteins is caused by the loss of 147 amino groups in the C-terminal region. The results of trypsin treatment and hydrophobic protein analysis revealed that this protein was mainly located on the cytomembrane of *M. bovis* and exposed on the cell surface. The protein dot blot assay confirmed that this 48 kDa protein can react with FN. As this was the first proteolytically processed *M. bovis* lipoprotein shown to interact with FN, it was designated as *M. bovis* fibronectin-binding lipoprotein (MbfN). Further experiments confirmed that the binding between MbfN and FN was dose-dependent, saturated adhesion could be achieved at a certain concentration, and anti-MbfN protein polyclonal antibody could specifically block this reaction. Interestingly, the authors found that the protein contains a consensus heparin binding sequence when analyzing it with bioinformatic analysis and then confirmed that MbfN indeed binds to heparin through a dot blot assay. These findings suggest that MbfN is an adhesin with two target proteins, for it can not only react with FN but also bind with heparin. Compared with the original strain, the adhesion ability of the mutant strain with the disrupted open reading frame of MbfN by transposon decreased significantly, and the

anti-MbfN antibody could significantly reduce the adhesion of *M. bovis* to MDBK cells.

Fructose-1,6-diphosphate aldolase

Xiang Gao and Jing Huang identified the adhesin activity of fructose-1,6-diphosphate aldolase (FBA) in 2018 and 2019, respectively (44, 45). FBA is a key enzyme in the process of glycolysis, gluconeogenesis and the Calvin cycle (46). In addition to energy metabolism, FBA also has many biological functions, including acting as Plg binding protein, transcription regulator and participating in host cell adhesion (47–51). FBA protein is composed of 291 amino acids with a molecular weight of 34 kDa. This protein lacks signal peptide or transmembrane region. The FBA is a highly conserved protein of mycoplasma, and the homology of FBA is up to 99% in *M. bovis*. The immunogenicity of FBA was determined by rabbit anti-*M. bovis* serum and it was also shown that the FBA was equally distributed in the cytoplasm and cell membrane. It was confirmed by western blotting and ELISA that the target protein of FBA was Plg. And the results of the adhesion inhibition experiment showed that the FBA antibody could block 34.4% of *M. bovis* adhesion to EBL

cells. On this basis, the Jing Huang team further confirmed that the protein can also bind to FN.

TrmFO

Yongpeng and colleagues discovered that a protein named methylenetetrahydrofolate tRNA⁻ (uracil-5⁻) -methyltransferase (TrmFO) has adhesin activity (52). The researchers carried out 150 and 180 generations of wild virulent strain HB0801 *in vitro* at 41°C to prepare a vaccine candidate with a certain protective effect. The genomic analysis found that the down-regulated proteins included NADH oxidase and variable lipoprotein VspX, which was involved in adhesion (53). Using an iTRAQ-based quantitative proteomic analysis, they also found that expression of TrmFO was down-regulated in the attenuated *M. bovis*-150 strain compared to the virulent strain HB0801. The molecular weight of TrmFO is 48.8 kDa and this protein contains 427 amino acids. It does not contain the signal peptide or transmembrane region according to the bioinformatic analysis results. The homology of TrmFO protein among different strains of *M. bovis* was more than 98%. TrmFO was identified as immunogenic by the positive serum of experimentally infected cattle and naturally infected cattle. Through the identification of 8 different isolates of *M. bovis*, it was found that this protein could be expressed in all of the experimental strains. Further immunological tests confirmed that it is a membrane-related protein, which is distributed in both the cytoplasm and cell membrane. The binding protein of TrmFO was identified to be FN by ligand dot blot and ELISA binding assay. The purified TrmFO can adhere to EBL cells, and the rabbit anti-TrmFO antibody significantly reduces the adhesion of purified TrmFO and *M. bovis* to this cell.

α - Enolase

The α -Enolase of a prokaryote is a highly conserved protein, which is mainly involved in many pathophysiological processes (54). In *M. bovis*, α -Enolase is about 49 kDa in size and contains 454 amino acids. This protein lacks classical protein-sorting signals but 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 (LYDENSCKKY). Zhiqiang and colleagues verified that this protein is present in both the membrane and the soluble cytosolic protein fractions of *M. bovis* cell by western blot. And they also confirmed by ELISA that α -Enolase can indeed bind to Plg in a dose-dependent manner, and this binding can be

inhibited by anti- α -enolase serum. Further, they confirmed that the adhesion of *M. bovis* to Plg pretreated EBL cells could be inhibited by rabbit anti- α -Enolase serum (55).

Mycoplasma immunogenic lipase A

Wawegama and colleagues identified a membrane protein with the potential to establish a detection method for *M. bovis*. This protein is about 226 kDa in size and has lipase activity. The researchers named it Mycoplasma immunogenic lipase A (MilA) (56). Adamu's team further studied this protein and found that MilA was composed of 3670 amino acids with a molecular weight of 303 kDa. After the protein is expressed, it is hydrolyzed into two fragments of 226 kDa and 50 kDa (57). Sequence analysis showed that MilA contains a glycosaminoglycan motif and multiple copies of a domain of the unknown function (DUF445), and the sequence contains two canonical binding motifs for heparin, "XBBXB" and "XBBBXXB". Trypsin treatment experiments confirmed that the 226 kDa protein was exposed to the surface of mycoplasma, which could bind 1-anilinonaphthalene-8-sulfonic acid, various lipids and heparin. MilA can also bind and hydrolyze ATP, indicating that the protein is likely to be a self-transporter. Antibodies against the carboxyl-terminal of MilA can inhibit the proliferation of *M. bovis in vitro*. The adhesion activity of MilA to heparin shows that the protein plays the role of adhesin, but unfortunately, the author has not conducted classical experiments such as adhesion and adhesion inhibition to further confirm the specific role of this protein in *M. bovis* adhesion.

p27 protein

It was found that a hypothetical lipoprotein named p27 with adhesin activity by Chen (58). The protein is 27.1 kDa in size and contains 241 amino acids encoded by the gene MBOV_RS03440. As a highly conserved protein, the amino acid homology of p27 between different *M. bovis* strains can reach 100%. It contains a signal peptide and 4 leucine-rich repeat regions (LRRs), but without transmembrane region. The full-length p27 gene could be amplified from the genomes of nine *M. bovis* strains by PCR with primers specific to MBOV_RS03440. And the 27 kDa protein was also specifically recognized by rabbit antiserum to p27 in all tested strains. ELISA assays revealed that recombinant p27 reacted with the sera of cattle naturally and experimentally infected with *M. bovis*. According to the western blot results, the p27 distribute in the whole cells, but most of the p27 molecules were surface exposed. IFA and specific serum blocking assays showed that p27 could directly adhere to EBL cells, and the

binding could be specifically blocked by anti-p27 serum. Further experiments confirmed that the target protein of p27 was FN, the interaction of them was direct and specific in a dose-dependent manner.

VpmaX

The genomic analysis of *M. bovis* Hubei-1 showed that the genome of this strain was missing the VSP gene cluster (59, 60). It was found that there is a gene annotated as VspA in the genome of *M. bovis* strain Hubei-1, but the coding protein of this gene is completely different from the VspA protein of PG45 typical strain. The author designated this gene as VpmaX and studied the adhesion of this gene (61). The VpmaX protein contains 229 amino acids with a molecular mass of approximately 35 kDa. Through bioinformatics analysis, it was found that the protein has a typical prokaryotic signal peptide and two repeat units, namely “KPSEQSGTNSQQGSG” and “QGSG”, in which the large unit repeated 3 times and the small one repeated 7 times. These structural characteristics are very similar to VSP family proteins. It was confirmed by an immunological method that the protein exclusively located in the cell membrane of *M. bovis*. The VpmaX protein which is expressed in prokaryotes can directly adsorb to EBL cells, and this adhesion to EBL cells can be blocked by anti-VpmaX serum. Interestingly, when the concentration of VpmaX is low, the protein is mainly distributed on the surface of EBL cells, while a higher concentration of VpmaX is used, it can enter the cytoplasm of EBL. Further experiments confirmed that the protein interacted with EBL cells in a dose-dependent manner, and the EBL cell membrane and cytoplasmic components could bind to VpmaX in this manner. Unfortunately, while the authors identified VpmaX as an adhesin, they did not identify the binding protein of this adhesin.

P26 protein

The process of discovering the adhesin P26 protein is anecdotal. As early as 1992, Evelyn and colleagues used the whole bacterial protein of the *M. bovis* J282 strain as an immunogen to screen monoclonal antibodies. Among them, a monoclonal antibody 4F6 that recognizes 26 kDa protein showed fantastic specificity. This monoclonal antibody reacts with all *M. bovis* strains selected in their study (62). In 1993, Sachs found that the adhesion of *M. bovis* to EBL cells can be specifically blocked by monoclonal antibody 4F6. This monoclonal antibody against 26 kDa protein can reduce the adhesion of *M. bovis* strains 120 and 454 to EBL cells by 46% and 70% respectively (63). Under non-blocking conditions, the

adhesion ability of 120 strains to EBL cells was higher than that of 454 strains. The results of western blot confirmed that the expression of P26 protein in the strain 120 was higher than that of the strain 454. These results suggested that P26 protein may play an important role in the adhesion of *M. bovis* to EBL cells. In 1995, Sachs purified P26 protein by HPLC and adsorbed EBL cells with P26 protein competing with *M. bovis* strain PG45, 120 and 454. It was found that a 1:100 ratio of P26 protein to the total protein of *M. bovis* could block 20% - 50% of *M. bovis* adsorption (64). Above results provide sufficient evidences that the P26 protein is an adhesin of *M. bovis*. Unfortunately, none of these publications provided the gene sequence or amino acid sequence of the P26 protein, nor identified the target protein of P26 protein.

Membrane surface variable lipoprotein family proteins

There are membrane surface variable lipoprotein family proteins (VSPs) in *M. bovis*. The members of this family mainly have the following features: (i) an N-terminal portion containing a prokaryotic lipoprotein signal sequence; (ii) a surface-exposed C-terminal region bearing extensive repetitive structures; (iii) high rates of spontaneous non-coordinate phase variation; (iv) high-frequency size variation; and (v) anchorage of these abundantly expressed amphiphilic proteins in the mycoplasma membrane via a lipid moiety at an N-terminal cysteine residue (65). Lysnyansky identified the gene cluster encoding the VSPs in the genome of *M. bovis* standard strain PG45 consists of 15 open reading frames, of which 13 encode VSPs. The research shows that all the amino ends of VSP protein have a conserved prokaryotic signal peptide, with more than 99% homology, and the VSP protein is also anchored on the cell membrane through the amino end (66). Thomas found it by blocking experiments that the monoclonal antibodies 9F1 and 2A8, which recognize VspC and VspF, can inhibit the adhesion of *M. bovis* to bovine bronchial epithelial (BBE) cells, and the monoclonal antibody 1E5 also has a certain blocking ability. These results indirectly suggested that VspC and VspF may be an adhesin (13). Sachse and colleagues conducted immunogenicity and adhesion studies on VspA, VspB, VspE and VspF of *M. bovis* (67). When the sequence analysis of the above VSPs, they identified that each sequence contained repetitive sequences. Artificially synthesized repetitive sequences or antibodies against these specific sequences was applied to conduct inhibit adhesion experiments of *M. bovis*, and the investigations showed that VspA, VspB, VspE and VspF reduced the adhesion of *M. bovis*, and monoclonal antibodies against VspA, VspB and VspF also inhibited the adhesion of *M. bovis* to host cells. The above results indicated that VspA, VspB,

VspC, VspE and VspF are *M. bovis* adhesins. It is worth noting that some monoclonal antibodies which inhibit the adhesion of *M. bovis* to EBL cells could not inhibit the adhesion to BBE cells (13). This result suggests that the adhesins of *M. bovis* against distinct host cells may be different. It is also a pity that neither of the two studies identified the target proteins of these VSPs adhesins.

Mbov_0503 coding protein

Through the screening of transposon mutant strains, Zhu and colleagues identified an adhesin encoded by the Mbov_0503 gene. This adhesin of *M. bovis* contains 548 amino acids. They carried out a prokaryotic expression of the adhesin and obtained a soluble protein with a molecular weight of 59.4 kDa (68). In that research, they first established the transposon mutation library for *M. bovis* HB0801 and screened 9 strains with decreased adhesion in MDBK cells and EBL cells, among which mbov_0503 protein mutant strain has the most stable characteristics, and supplement of mbov_0503 can restore the adhesion ability of mutant. Further analysis found that mbov_0503 protein on the surface of *M. bovis*. The results of laser scanning confocal microscopy and ELISA confirmed that Mbov_0503 protein could directly adhere to the surface of EBL cells and bind to EBL cell membrane proteins in a dose-dependent manner. The researchers found that the adhesin could only partially affect the adhesion of *M. bovis* to host cells and that the mutation had no effect on the proliferation of the mutant, but the deletion of Mbov_0503 resulted in a significant decrease in the ability of the mutant to cross the cell barrier. Although not verified in animal experiments, this adhesion is likely to be closely related to the pathogenicity of mycoplasma. Furthermore, the ligand for Mbov_0503 was not identified in this study.

24 kDa proteins

As mentioned above, Thomas demonstrated that *M. bovis* can adhere to bovine tracheal epithelial cells (BBE) (13). Their follow-up study found that the adhesion ability of *M. bovis* 2610 strains decreased significantly after passage *in vitro*. The results of 2D electrophoresis analysis showed that the expression of a protein with a size of about 24 kDa was significantly reduced in the high passage strain. The sequence of this protein was obtained by LC-MS/MS analysis, but it does not match any of the known sequences in the *M. bovis* database by BLAST software. Trypsin digestion experiments showed that the 24

kDa protein was a membrane protein, and serum or monoclonal antibody against this protein could reduce the adhesion of 2610P7 (passage 7 *in vitro*) strain to BBE cells. According to the above observations, this protein with a size of 24 kDa is an adhesin (69).

Adhesin processing

Post-translational processing of adhesin is a relatively common phenomenon, including signal peptide excision and post-translational hydrolysis. So far, this phenomenon has been found in many pathogens, such as *M. pneumoniae*, *M. hyopneumoniae*, *Streptococcus* bacteria, some parasites, etc. The processing phenomenon of classical adhesin p97 and P102 family proteins of *M. hyopneumoniae* is universal. In *M. hyopneumoniae*, it was not only that multiple adhesins have such post-translational processing, but also adhesin-related proteins (70). Post-translational processing is essential for the colonization, adhesion and pathogenicity of *M. hyopneumoniae* (71, 72). And the adhesin protein of *M. hyopneumoniae* often shows multiple cleavage sites, resulting in different cleavage products showing different adhesion characteristics. For example, the p123j protein of *M. hyopneumoniae* has cleavage events at multiple sites, and the product of this protein can bind to diverse host cell surface components (73). Another mycoplasma that has fully demonstrated the phenomenon of adhesin processing is *M. pneumoniae*. Studies have shown that almost half (317; 46%) of ORFs derived from *M. pneumoniae* strain M129 are post-translationally modified (74). *M. pneumoniae* needs a complex attachment organelle to achieve adhesion and mobility functions, and its main components include adhesins P1 and P30. The major adhesins, P1 and P30, are localized to the tip of the attachment organelle by the surface-accessible cleavage fragments P90 and P40 derived from Mpn142 (75). In bacteria, the treatment of adhesion proteins is also crucial. For example, *Streptococcus* adhesion protein AbpA needs the treatment of SrtB to have the ability to adhere to salivary amylase. The absence of SrtB brings about AbpA to be released into the culture supernatant, and *Streptococcus* loses the ability to bind salivary amylase (76). In addition, this post-translation processing of adhesin is also identified in parasites (77). In *M. bovis* adhesins, post-translational processing has also been confirmed (43, 57), but the research on this phenomenon is not in-depth. The treatment of adhesin may be closely related to the exposure of adhesin binding sites, immune escape, invasion and toxicity differences. Considering this, this part of the work needs to be increased in the study of *M. bovis* adhesins in the future.

Biological functions of adhesins and their target proteins

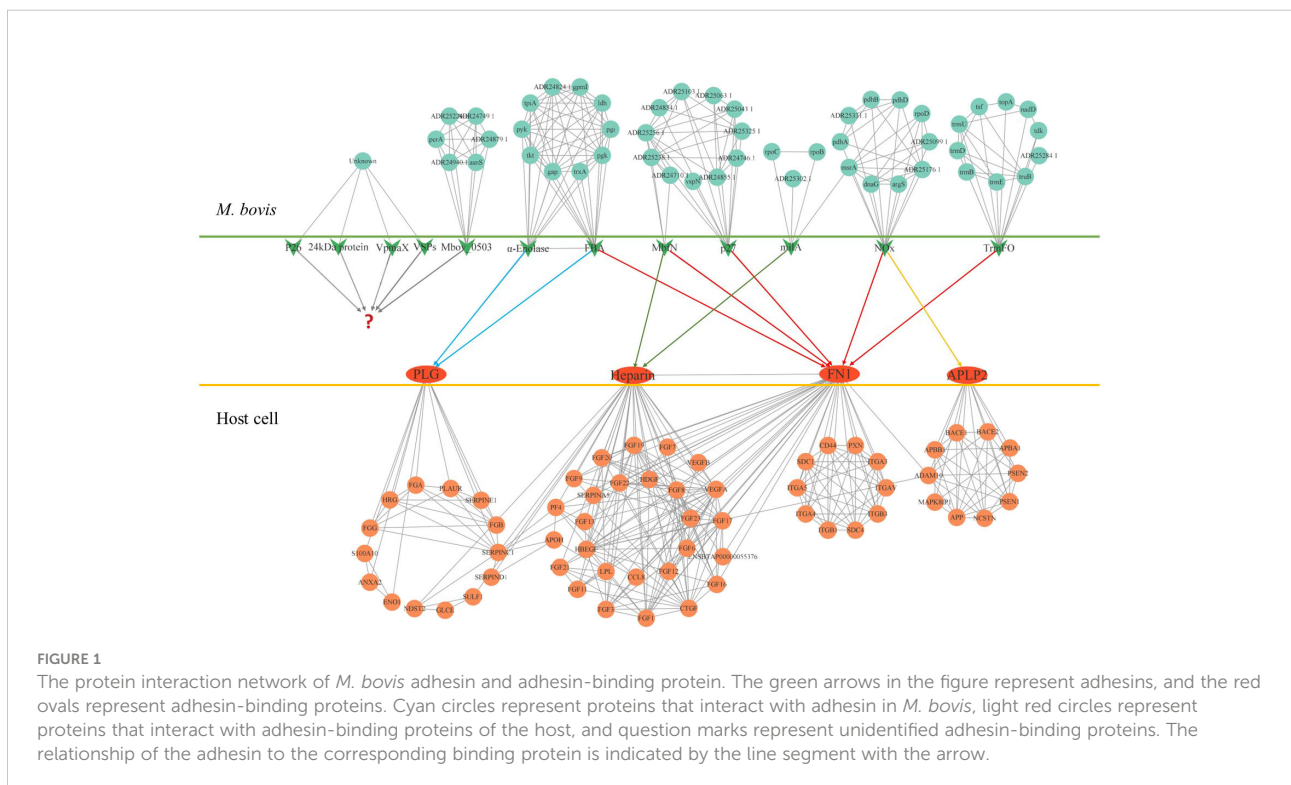
Limited by the information obtained from existing studies, we know little about the role of *M. bovis* adhesin's effect on host cells and itself in infection. Based on the reported adhesins of *M. bovis*, host binding proteins and STRING database, all the interacting proteins was shown in Figure 1. Among these adhesins, the relative proteins of FBA and α -Enolase are highly coincident, and the interaction proteins of MbfN also belong to the interaction protein network of p27, which may mean that these adhesins have the same or similar physiological effects on *M. bovis* in the process of infection, and this physiological effect may be more important for the survival of *M. bovis* in specific host tissues. Unfortunately, we were still unable to obtain the interaction protein information of P26, VSPs, VpmaX and 24 kDa protein. To adhesin binding proteins, the proteins that interact with FN1 were divided into two groups, one of which was highly coincident with heparin-related proteins. Further, according to the database KOBAS, we found that the adhesin-related pathways maybe associated with biological activities such as biosynthesis and energy metabolism. *M. bovis* infection cause a series of biological reactions in the host cells, which may promote the pathogen colonization or adverse effects to the host. Based on the DAVID database information, the adhesin-binding proteins were not only related to various cancer signaling pathways proteins, but

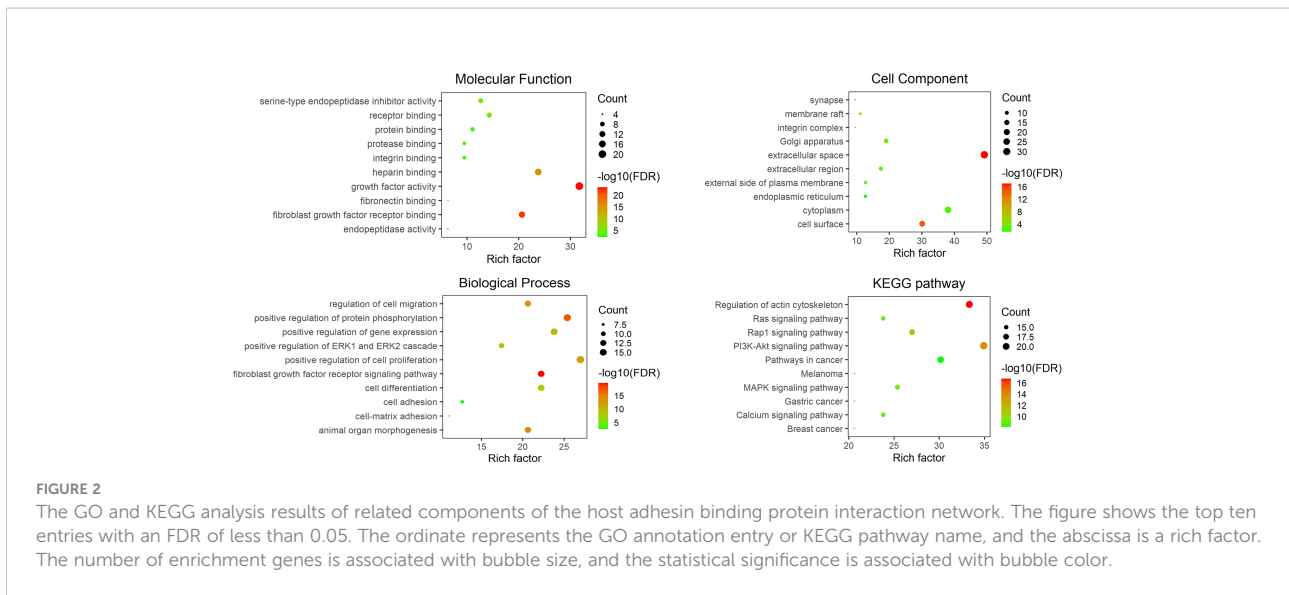
also involved in cell adhesion, connectivity, apoptosis, inflammatory responses and many other processes (Figure 2).

Although it is not easy to define the biological function of adhesin on *M. bovis* itself, this part of work is indeed an important aspect of *M. bovis* adhesin research. At the same time, the effect of adhesin on the host also needs further verification, especially the protein interaction network and signal pathway related to pathogenicity. At present, the study is very rare to independently verify the effect of *M. bovis* adhesin on host cells, but such research is of great significance for advancing the research of *M. bovis* pathogenicity and vaccines.

Application potential of adhesin in vaccine development

Because the antibiotic treatment is not working as expected (78), an effective vaccine has become one of the desirous means expected to prevent and control the disease. In recent years, magnitude work has focused on the terms of *M. bovis* vaccine research. Scientists have made attempts in inactivated vaccines, attenuated vaccines and subunit vaccines. Unfortunately, they have not achieved ideal results (79). As mentioned above, most adhesins of *M. bovis* are membrane surface proteins, with excellent antigenicity, and play critical roles in infection, pathogen proliferation and host damage. Therefore, these proteins have great potential in the *M. bovis* vaccine developing.





The problems faced by the development of the *M. bovis* subunit vaccine are mainly the following aspects: 1. Some candidate proteins may be protective but lack conservatism. Such as VSPs, are considered to be potential vaccine candidate proteins. However, the antigenic variation in these lipoproteins may make the vaccines ineffective in the long run (67); 2. Some candidate components not only failed to provide protection but will aggravate clinical signs, such as higher lesions in lung injury (80); 3. It is insufficient available selection space for high-quality vaccine candidate protein. Although a variety of vaccines containing single protein or multi-protein have been exploited, the expected effect has not been achieved. Researchers have tried a variety of proteins, including glyceraldehyde-3-phosphate dehydrogenase alone as a vaccine (80, 81), PdhA, PepA, Tuf, P48, P81, OppA, LppA, PepQ, O256 and DeoB combination as a vaccine (82), and *M. bovis* membrane fractions and cell extracts, etc. (83). In fact, given the role of *M. bovis* adhesin in its infection, this kind of protein may be more suitable for vaccine research and development. When combined with reverse vaccinology and structural biology, it may have a better prospect. For example, the α -enolase of *M. bovis* can be expressed on the surface of the pathogen and combined with plasminogen. These characteristics are very similar to the α -enolase of *Streptococcus suis*, which shows good protection in mice and zebrafish models (84, 85). It is believed that with the help of emerging technologies and the deepening of adhesin research, a new situation will be opened in the field of vaccine research.

Conclusion

Although some progress has been made in the study of bovine adhesins, there is still much room for improvement in

this field. The research of *M. bovis* adhesin is relatively backward in the whole field of mycoplasma. The shortcomings are mainly as follows: 1. The techniques of adhesin identification need to be further improved. In addition to the methods mentioned in this review, other technologies such as IP/Co-IP, mass spectrometry, proteomics, and bioinformatics can also be used to identify adhesin; 2. The research on the mechanism of the function realization of adhesins is not deep enough, especially on the post-translational processing; 3. Does *M. bovis* adhesin require the involvement of chaperones, and if so what are these chaperones and how do they work? These issues also need to be clarified; 4. The difference of *M. bovis* adhesin in different host cells needs further study, which is of great significance to further explain the infection of *M. bovis* to multiple organs and tissues; 5. In addition to adhering to host cells, the question of what effect does bovine mycoplasma adhesin on host cells has not been well clarified. In this review, based on the identified *M. bovis* adhesins, adhesin-binding proteins and online database, we discussed the possible biological effects of the adhesins on the pathogen and host cells. These possible biological effects have not been verified, but are inferred from available information. Strictly, the support of the existing literature and arguments in this part of the discussion is not sufficient. On one hand, this discussion hopes to attract the attention of relevant researchers, and on the other hand, it also hopes to provide limited background knowledge for such research.

Many excellent researchers and teams have made outstanding contributions to the research of *M. bovis* adhesins, and more than 10 adhesins have been identified. Adhesins have special significance in the process of *M. bovis* infection, and some adhesins are very well-conservative and have excellent immunogenicity. Many adhesins have a variety of biological activities, which may be related to the virulence of *M. bovis*. Therefore, the study of *M.*

bovis adhesin may be of great benefit to the progress of serological detection methods, vaccines, and pathogenesis of *M. bovis*.

Author contributions

QX, QP and QW writing-original draft; QX and JX designed and finally revised the article. All authors contributed to the article and approved the submitted version.

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