The *Mycoplasma gallisepticum* OsmC-like protein MG1142 resides on the cell surface and binds heparin

Cheryl Jenkins,1,2 Steven J. Geary,3,4 Martha Gladd3,4 and Steven P. Djordjevic1

1Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Camden, NSW 2570, Australia
2School of Biological Sciences, University of Wollongong, NSW, Australia
3Center of Excellence for Vaccine Research, The University of Connecticut, Storrs, CT 06269-3089, USA
4Department of Pathobiology and Veterinary Science, The University of Connecticut, Storrs, CT 06269-3089, USA

*Mycoplasma gallisepticum* is an avian pathogen that causes a chronic respiratory disease of chickens and results in significant economic losses to the poultry industry worldwide. Colonization of the host and the establishment of chronic disease are initiated by the cytadherence of *M. gallisepticum* to the host respiratory epithelium. While several proteins involved in cytadhesion have been characterized, molecules that interact with components of the host extracellular matrix, a process that is central to pathogenesis, are only now being identified. In this study, *M. gallisepticum* whole cells were shown to bind heparin in a specific and saturable manner. Heparin also significantly inhibited the binding of *M. gallisepticum* to the human lung fibroblast cell line MRC-5, suggesting a potential role for glycosaminoglycans (GAGs) in cytadherence. *M. gallisepticum* protein MG1142 (encoded by *mga_1142*), which displays homology to the osmotically induced (OsmC) family of proteins, binds strongly to heparin, is highly expressed during *in vitro* culture, and is surface accessible. Recombinant MG1142 bound heparin in a dose-dependent and saturable manner with a dissociation constant ($K_d$) of $10 \pm 1.8$ nM, which is within a physiologically significant range, compared to that of other heparin-binding proteins. Binding to heparin was inhibited by the heavily sulfated polysaccharide fucoidan, but not by mucin or chondroitin sulfate A or B, suggesting that electrostatic interactions between the sulfate groups of heparin and the positively charged basic residues of the MG1142 protein are important in binding. The ability of *M. gallisepticum* to bind GAGs may contribute to host adherence and colonization.

INTRODUCTION

*Mycoplasma gallisepticum* is a widespread avian pathogen that is associated with a chronic respiratory disease in chickens, and is the primary agent of infectious sinusitis in turkeys (Jordan, 1979). *M. gallisepticum* cells adhere to and colonize the host respiratory epithelium, establish chronic infections, and leave the host vulnerable to secondary assaults. In chickens, *M. gallisepticum* infections are characterized by tracheitis and air sacculitis, and in the presence of concurrent bacterial or viral infections, they can result in high morbidity and mortality (Jordan, 1979). *M. gallisepticum* infections are communicable via aerosols, allowing the organism to spread rapidly through commercial flocks, while transovarial transmission can result in decreased egg production (Ley & Yoder, 1997). Antimicrobial therapies are of limited use in controlling outbreaks of this disease, and the emergence of drug-resistant strains can render some of these ineffective (Gautier-Bouchardon et al., 2002). Economic losses to the poultry industry are significant (Levisohn & Kleven, 2000).

*M. gallisepticum* pathogenesis is a complex, multifactorial process. While the respiratory tract is the primary site of *M. gallisepticum* colonization, disease can spread systemically, creating multiple infection loci. Thus, polyarthritis (Lamas da Silva & Adler, 1969), salpingitis (Domermuth et al., 1967; Nunoya et al., 1997) and encephalopathy (Chin et al., 1991) have all been observed as sequelae of *M. gallisepticum* infections.

Abbreviations: ECM, extracellular matrix; GAG, glycosaminoglycan; HBHA, heparin-binding haemagglutinin.
infection. Recent studies have demonstrated that minimally passed \textit{M. gallisepticum} strain R\textsubscript{low} is able to invade non-phagocytic cells \textit{in vitro} (Winner \textit{et al.}, 2000). \textit{In vivo} studies using R\textsubscript{low} have shown that this strain is able to induce air sac lesions and disseminate from the respiratory tract to secondary infection sites. In contrast, highly passed strains (R\textsubscript{high}) are non-invasive, but are still able to adhere to and colonize the trachea (Much \textit{et al.}, 2002). These findings indicate that \textit{M. gallisepticum} uses distinct mechanisms to adhere to the respiratory tract and to disseminate within the host.

The co-expression of two molecules, GapA and CrmA, has been shown to be essential for cytadherence \textit{in vitro} and the production of air sac lesions \textit{in vivo} (Papazis \textit{et al.}, 2002). R\textsubscript{high} is known to lack both GapA and CrmA (Papazis \textit{et al.}, 2000). Despite this, complementation of R\textsubscript{high} with wild-type \textit{gapA} and \textit{crmA} does not completely restore virulence, indicating that \textit{M. gallisepticum} pathogenesis is complex. Furthermore, while a number of adherence factors have been characterized, little is known about the host ligands with which \textit{M. gallisepticum} interacts.

For many pathogens, interactions with components of the host extracellular matrix (ECM) are central to bacterial adherence and pathogenesis. Proteins that interact with the host ECM are found in a diverse array of pathogens, including several \textit{Mycoplasma} species (Alvarez \textit{et al.}, 2003; Burnett \textit{et al.}, 2006; Dallo \textit{et al.}, 2002; Giron \textit{et al.}, 1996; Jenkins \textit{et al.}, 2006; Kannan \textit{et al.}, 2005; May \textit{et al.}, 2006). By presenting specific receptors for ECM components such as fibronectin, laminin and glycosaminoglycans (GAGs) on their surfaces, pathogens are able to mediate interactions with (Menozzi \textit{et al.}, 2002; Patti & Hook, 1994), and in some cases invade (Dubreuil \textit{et al.}, 2002; Joh \textit{et al.}, 1999; Pethe \textit{et al.}, 2000), host cells. Binding to exogenous GAGs (e.g. heparin, heparan sulfate and chondroitin sulfate) may play a particularly important role in bacterial pathogenesis. Recruitment of these compounds to the bacterial cell surface has been shown to facilitate bridging interactions with other ECM components and inflammatory factors, which are themselves GAG-binding proteins. Exploiting these interactions may allow the infectious agent to invade host cells, modulate the inflammatory response, and evade the host immune system (Duening \textit{et al.}, 1999).

In this study, we demonstrated that \textit{M. gallisepticum} strain R\textsubscript{low} bound heparin (a heparan sulfate analogue commonly used for identifying GAG-binding proteins), and investigated the role of this molecule in interactions between \textit{M. gallisepticum} and the lung fibroblast cell line MRC-5. The availability of a genome sequence for \textit{M. gallisepticum} strain R (Papazis \textit{et al.}, 2003) facilitated the identification of heparin-binding proteins via a proteomic approach. One such protein is a 14.5 kDa OsmC-like molecule (MG1142) that is highly expressed and resides on the cell surface. The ability of \textit{M. gallisepticum} to bind GAGs may contribute significantly to the pathogenesis of this organism.

### Methods

#### Bacterial strains and culture.

\textit{M. gallisepticum} (strain R\textsubscript{low}) was cultured in Hayflick’s medium (Thorns & Boughton, 1980) at 37°C from 1 ml frozen stocks of exponential-phase cultures which were maintained at −70°C.

#### 2D gel electrophoresis.

A 250 ml culture of \textit{M. gallisepticum} (strain R\textsubscript{low}) was harvested at 10 000 r.p.m. for 30 min in a Sorvall RC5C Plus centrifuge using a GSA rotor. The cells were washed in PBS, pH 7.2, resuspended in standard sample solubilization buffer (40 mM Tris, 8 M urea, 100 mM DTT, 4 % w/v CHAPS, 0.8 % carrier ampholytes), and sonicated using the cup horn attachment on a Microson Ultrasonic cell disruptor (Misonix). The samples were ultracentrifuged in a TL100 ultracentrifuge (Beckman Coulter) for 1 h at 50 000 r.p.m. using a TL1A00.3 rotor, and the supernatant was reserved for analysis. For 2D electrophoresis, 200–300 μg of whole-cell protein was used to rehydrate each IEF ReadyStrip (linear pH 3–10) (Bio-Rad Laboratories) for 6 h at room temperature. The IEP strips were focussed using a Protein IEF cell (Bio-Rad Laboratories) at 100, 300, 600, 1000 and 2000 V, with each step lasting 1 h for 11 cm IEF strips, and 2 h for 17 cm strips. For the final focussing step, the strips were subjected to 4000 V for 40 V h (11 cm strip) or 60 000 V h (17 cm strip). For the second dimension, the IEP strips containing the separated proteins were used to load 12 % polyacrylamide gels, which were then electrophoresed at 3 W per gel for 30 min, followed by 40 W for 1 h and 80 W for 5 h. The gels were then either stained with colloidal Coomassie Blue (17 %, w/v, ammonium sulfate; 3 %, v/v, phosphoric acid; 0.1 %, w/v, Coomassie G-250; 34 %, v/v, methanol), or Western-transferred for immunoblot or ligand blot analysis.

#### Ligand blotting.

2D ligand blots were performed on Hybond-P PVDF membranes (Amersham Biosciences). Following Western transfer, the membranes were blocked for 1 h in 5 % (w/v) skimmed milk/PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4). Blots were reacted for 1.5 h with biotinylated heparin (Sigma, catalogue no. B9806) which had been diluted in wash buffer (0.1 %, w/v, skimmed milk/PBS) to a final concentration of 10 μg ml\textsuperscript{−1}. The membranes were then washed three times for 10 min in wash buffer, and reacted with a 1:3000 concentration of streptavidin peroxidase (Roche). The membrane was washed as before, equilibrated in 100 mM Tris/HCl (pH 7.6) and developed with a solution of 3,3'-diaminobenzidine (DAB) (Sigma). Peptide mass fingerprinting of a 14.5 kDa heparin-reactive protein (MG1142) was performed on tryptic digests of the protein spot excised from a 2D gel, as previously described (Djordjevic \textit{et al.}, 2004). For 1D ligand blots, \textit{M. gallisepticum} cells were harvested, heated in Laemmli buffer, and electrophoresed on a 12 % polyacrylamide gel. Proteins were transferred and blotted as described above for 2D ligand blots, except that blots were reacted with either 10 μg biotinylated heparin ml\textsuperscript{−1} or 10 μg biotinylated heparin ml\textsuperscript{−1} plus a one-, 10- or 50-fold excess of unlabelled heparin.

#### DNA extraction and PCR.

DNA was extracted from 6 ml overnight cultures of \textit{M. gallisepticum} (strain R\textsubscript{low}) using Instagenic matrix (Bio-Rad Laboratories) according to the manufacturer's instructions. The gene corresponding to the MG1142 protein was amplified from \textit{M. gallisepticum} genomic DNA by PCR. PCR primers were designed to enable directional cloning into the pET100-TOPO vector (Invitrogen) and the incorporation of an N-terminal polyhistidine tag. TGA codons encode tryptophan in mycoplasmas, and expression in \textit{Escherichia coli} results in truncated products due to premature stops. To facilitate expression of the gene in \textit{E. coli}, a long reverse primer (45-mer) was designed to incorporate a mismatch, correcting a TGA codon at amino acid position 141 to a TGG codon. Primer sequences were as follows (mismatch shown in bold): forward primer, 5'-CACC ATT TAC GAA AGG ATA TTA TGT G-3'; reverse primer, 5'-TTA GTA AGG AAC GTC CAT TCC GTT AAC CTT TAA GCA GAT GAT

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TTG-3'. The PCR reaction was carried out in a 50 μl volume using the proofreading polymerase Pwo (Roche). Reactions contained 100 ng template DNA, 1 x Pwo PCR buffer containing MgSO4, 0.2 mM dNTPs, 200 nM primers and 2.5 U polymerase.

Cloning and expression. The PCR product was purified using the QIAquick PCR Purification kit (Qiagen), ligated into the pET100D-TOPO vector (Invitrogen), and transformed into chemically competent E. coli TOP10 cells (Invitrogen). Plasmids were extracted from transformants using the QIAprep MiniPrep kit (Qiagen), and screened by restriction digest with PstI (Fermentas). The plasmid construct was confirmed via DNA sequencing.

Protein expression and purification. The pET100D:MG1142 construct was transformed into the E. coli expression strain BL21 Star (DE3) (Invitrogen), and the 14.5 kDa protein expressed as a hexahistidyl fusion. E. coli BL21 Star cells were grown to mid-exponential phase and induced with 1 mM IPTG for 4 h. Recombinant protein was purified under denaturing conditions as described previously (Jenkins et al., 2006), and was dialysed for 48 h against PBS/0.1 % SDS or PBS/5% (v/v) glycerol to remove the urea. The concentration of the purified proteins was estimated using the Bradford assay (Bio-Rad Laboratories).

Production of polyclonal antiserum. Polyclonal antiserum to purified recombinant MG1142 protein was generated as described previously (Jenkins et al., 2006). Aliquots of serum for subsequent use in immunogold-labelling experiments were purified using the Nab Protein A Spin Purification kit (Pierce) according to the manufacturer’s instructions.

Western blotting. The purified recombinant MG1142 was electro-phoresed on an SDS-polyacrylamide gel, as described previously (Djordjevic et al., 2004). The protein was transferred onto a PVDF membrane, blocked with 5 % skimmed milk/PBS, and reacted with MG1142 antiserum (1:1000). The membrane was washed three times for 10 min in 0.1 % skimmed milk/PBS, and reacted with a sheep anti-rabbit horseradish peroxidase (HRP) conjugate (Chemicon) at a concentration of 1:1500. The membrane was then washed three times for 10 min in 0.1 % skimmed milk/PBS, equilibrated in 100 mM Tris/HCl (pH 7.6), and developed with a solution of DAB (Sigma).

Whole-cell dot blotting. The protocol for the whole-cell dot blotting was adapted from an earlier study (Mitchell et al., 2004). A 6 ml overnight culture of M. gallisepticum was harvested, washed and resuspended to OD600 2.0 in sterile PBS. Serial twofold dilutions of whole cells (in 10 μl volumes) were spotted onto a Hybond-C super nitrocellulose membrane using a Bio-Dot Microfiltration apparatus (Bio-Rad Laboratories). After spotting, the membrane was blocked for 1 h in 5 % skimmed milk/PBS, and then reacted for 1 h with a 1:500 dilution of anti-MG1142 antiserum. The membrane was then washed three times for 10 min in 0.1 % skimmed milk/PBS, and reacted with a sheep anti-rabbit horseradish peroxidase (HRP) conjugate (Chemicon) at a concentration of 1:1500. The membrane was then washed three times for 10 min in 0.1 % skimmed milk/PBS, equilibrated in 100 mM Tris/HCl (pH 7.6), and developed with a solution of DAB (Sigma).

Immunogold labelling of M. gallisepticum whole cells. M. gallisepticum cells were harvested from a broth culture by centrifugation, and washed twice and resuspended in PBS. Parlodion/carbon-coated 300 mesh nickel grids (ProSciTech) were floated on drops of the cell suspension for 2 min in a moist Petri dish. The grids were incubated for 1 min in a phosphate buffer solution (pH 6.8) containing 1 % (w/v) BSA, 0.5 % (v/v) Tween 20 and 0.02 % (w/v) sodium azide, and then transferred to drops of MG1142-specific or pre-immune serum (negative control) for 90 min at 37 °C. After three 5 min washes in phosphate buffer, the grids were floated for 45 min on drops of protein A-gold (15 nm; BBInternational), which had been diluted 1:50. The grids were washed in phosphate buffer and then MilliQ water, stained with a 2 % aqueous solution of uranyl acetate (Merck), and blotted dry. Grids were examined using a Philips 208 transmission electron microscope.

Heparin-binding assays. The ligand dot blot assay was performed as previously described (Jenkins et al., 2006), using a Bio-Dot Microfiltration apparatus (Bio-Rad Laboratories) and 0.5 μg (100 μl volume) protein per well. A duplicate blot was stained with amido black protein stain (0.1 %, w/v/1 amido black, 7 %, v/v/1 acetic acid) immediately after spotting to ensure that equal amounts of protein had been transferred to the membrane.

Microtitre plate binding assays were performed using plates with a 96-well format (Linbro/Titertek; ICN Biomedicals). For whole-cell assays, a 125 ml exponential-phase culture was harvested, washed with PBS, and fixed in 1 % (w/v) paraformaldehyde/PBS. The cells were coated onto a microtitre plate by diluting them to OD600 0.4 with carbonate coating buffer (18 mM NaHCO3, 27 mM Na2CO3, pH 9.5) and then centrifuging them in a CS-6 benchtop centrifuge (Beckman Coulter) at 2000 r.p.m. for 10 min.

For assays using purified protein, plates were coated overnight in a humidified chamber with 1 μg MG1142 per well, which had been diluted in 100 μl carbonate coating buffer. Assays using whole cells and purified protein were both performed as described previously (Jenkins et al., 2006), except that the final concentrations of biotinylated heparin were 0, 0.1, 1, 10, 20, 50, 100 and 200 μg ml−1 for the whole-cell assay and 0, 0.01, 0.1, 1, 10, 20, 50 and 85 μg ml−1 for assays employing recombinant protein. To determine the specificity of the interaction between MG1142 and heparin, we repeated the protein assay, but pre-mixed a 50-fold excess of heparin (Sigma, catalogue no. H-3149) with the biotinylated heparin prior to addition to the plates. Competitive binding assays were also performed as above, but with the addition of a one-, two-, five-, 10- and 30-fold excess of inhibitor pre-mixed with the biotinylated heparin. The inhibitors employed were all sulfated polysaccharides and included unlabelled heparin, fucoidan, mucin and chondroitin sulfate A and B (Sigma). All microtitre plate binding assays were performed in triplicate and incorporated a range of controls in which wells were uncoated, and no biotinylated heparin or streptavidin/peroxidase was added. To ensure efficient coating of proteins to the microtitre wells, an additional control, using protein-specific antiserum and HRP-labelled anti-rabbit IgG (Chemicon), was included. The resulting data were plotted with GraphPad Prism version 4.02 for Windows (GraphPad Software) using nonlinear regression.

MRC-5 adherence assay. Adherence assays were performed with the human lung fibroblast cell line MRC-5, which has been used previously as a model for cell adherence (Geary et al., 1989). M. gallisepticum Rsub was labelled with [methyl-3H]thymidine (NEN/PerkinElmer) at 10 μCi ml−1 (370 kBq ml−1) and grown to mid-exponential phase in Hayflick’s broth, complete with 10 % (w/v) horse serum and 5 % (w/v) yeast extract, at 37 °C. Labelled M. gallisepticum was centrifuged at 8000 g for 15 min, then washed twice with 35 ml PBS per wash, and resuspended in one-tenth of the original volume. MRC-5 human lung fibroblasts (provided by the University of Connecticut Animal Cell Culture Facility) were seeded at 1 x 105 cells per well in 24-well tissue-culture plates (Corning Life Sciences) in DMEM with 10 % (v/v) fetal bovine serum and grown to 98–100 % confluence at 37 °C in 5 % CO2. The medium was aspirated from the MRC-5 monolayers, and the cells washed twice with 1 ml PBS per wash. Test wells received 200 μl per well of various concentrations of heparin [sodium salt, grade-1A from porcine intestinal mucosa (Sigma)] prepared in PBS, followed immediately by the addition of 200 μl [3H]-labelled M. gallisepticum which were then gently triturated to achieve the desired final concentrations. Control wells consisted of MRC-5 monolayers with PBS alone (negative control), and MRC-5 monolayers
with \(^{3}H\)-labelled *M. gallisepticum* without heparin (positive control). The cells were then incubated at 37 °C in 5% CO\(_{2}\) for 60 min. The supernatants were aspirated, and the MRC-5 cells were washed twice with 1 ml PBS per wash. To dissociate the cells, 500 μl 0.05 M NaOH was added to each well. The contents of each well were then transferred to scintillation vials containing 5 ml Cytoscint (Fisher Scientific). The samples were counted in a Beckman LS3801 liquid scintillation counter (Beckman Coulter). All data points were obtained in quadruplicate. Statistical analysis of data from MRC-5 adherence assays was performed using all-pairwise multiple-comparison one-way analysis of variance, as implemented in SigmaStat version 3.11 (Systat Software).

**RESULTS**

*M. gallisepticum* cells bind heparin

Whole *M. gallisepticum* cells were coated onto microtitre plates and assayed for heparin-binding activity. *M. gallisepticum* was found to strongly bind heparin in a specific and saturable manner (Fig. 1a). Furthermore, adherence of \(^{3}H\)-labelled *M. gallisepticum* to the human fibroblast cell line MRC-5 was inhibited by ~33% in the presence of heparin (saturating concentration of 125 μg ml\(^{-1}\)) (Fig. 1b). This inhibitory effect was found to be significant relative to the 0 μg ml\(^{-1}\) heparin control (*P*, 0.001). 1D ligand blots of whole-cell proteins indicated that there were several *M. gallisepticum* proteins able to interact with biotinylated heparin (Fig. 1c, lane 1); however, competitive inhibition of heparin binding (as indicated by a decrease in the intensity of the protein band, with increasing concentrations of unlabelled heparin relative to biotinylated heparin) (Fig. 1c, lanes 2–4) was only observed for some of these proteins (large arrows). Bands that did not display competitive inhibition (small arrows) were assumed to represent proteins that interacted with heparin in a non-specific manner. Of the proteins that were observed to interact with heparin in a specific manner, only those that reside on the cell surface and are therefore biologically available to bind heparin were considered to be of interest. For this reason, we sought to identify specific proteins.

**Fig. 1.** (a) Binding of biotinylated heparin to freshly cultured and immobilized *M. gallisepticum* cells. Total binding (∙), specific binding (△) and non-specific binding (○) are shown. The total binding was determined using a standard microtitre plate assay, while the non-specific binding was determined in the presence of a 50-fold excess of unlabelled heparin. The specific binding curve was generated by subtracting the non-specific binding from the total binding. Error bars indicate SD for triplicate readings. (b) Adherence of *M. gallisepticum* to MRC-5 cells. Partial inhibition of adherence (~33%) was seen in the presence of heparin up to a saturating concentration of 125 μg ml\(^{-1}\). The inhibitory effect was significant relative to the no-heparin control (*P*, 0.001), and was observed over four replicate experiments. (c) 1D ligand blots of *M. gallisepticum* whole-cell proteins probed with biotinylated heparin (lanes 1–4) and inhibited with a one- (lane 2), 10- (lane 3) or 50-fold (lane 4) excess of unlabelled heparin. Protein bands that bound heparin in a specific manner (i.e. decrease in intensity with an increase in the concentration of unlabelled heparin from lanes 1–4) are indicated by large arrows. Small arrows indicate proteins that appeared to react with heparin non-specifically. Molecular mass marker sizes are in kDa.
within this organism that might be responsible for heparin-binding activity and could be localized to the cell surface.

**Preliminary identification of MG1142**

2D electrophoresis and ligand blotting were used to achieve adequate separation of *M. gallisepticum* whole-cell proteins and to identify which proteins were capable of binding heparin. MG1142 was initially identified as a highly expressed, low-molecular-mass protein (14.5 kDa) in these proteomic analyses. The 14.5 kDa protein was shown to react with heparin in the 2D ligand blots (Fig. 2a), and the corresponding protein spot was subsequently excised and subjected to peptide mass fingerprinting. The resulting peptide matches (Fig. 2b) corresponded to the predicted amino acid sequence derived from the *mga_1142* gene.

The *mga_1142* gene was annotated in the recently sequenced *M. gallisepticum* strain R genome as encoding an OsmC-like protein (osmotically inducible protein C) (GenBank accession no. NP_853069). Such proteins are described in the literature as representing ‘putative envelope proteins’, suggesting that MG1142 might be surface-located. As MG1142 represents one of the most highly expressed proteins in the *M. gallisepticum* proteome (data not shown), was seen to react strongly with heparin in 2D ligand blots, and was a good candidate for a surface protein, we proceeded to express and purify this molecule for further analysis.

**Generation of recombinant MG1142**

The gene for MG1142 was amplified (Fig. 3a) using sequence information derived from the *M. gallisepticum* strain R genome, and expressed as a polyhistidine fusion protein. DNA sequencing confirmed that the construct

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![Fig. 3.](http://mic.sgmjournals.org)
 contained mga_1142. 1D SDS-PAGE analysis of the purified protein revealed two bands with approximate molecular masses of 14 and 26 kDa (Fig. 3b). In an earlier study, the OsmC protein from Xylella fastidiosa migrated as two bands on SDS-PAGE gels, which represented different oxidation states of the molecule (Cussiol et al., 2003). In this study, treatment of MG1142 with DTT or hydrogen peroxide was not shown to influence the occurrence of two bands (data not shown). Therefore, the 26 kDa band was assumed to be a dimeric form of the protein, since it was approximately double the mass of the low-molecular-mass band. As shown in immunoblots, antibodies raised against the recombinant MG1142 protein reacted strongly with both the high- and low-molecular-mass bands, although notably, only the 14 kDa band was found to be present in M. gallisepticum whole-cell extracts (Fig. 3c). We excised both bands from a Coomassie-stained gel and analysed them using peptide mass fingerprinting. The data confirmed that the peptide profile of the 14 kDa band matched that of MG1142. Furthermore, the peptide matches from the 26 kDa band were identical to those from the 14 kDa band, indicating that the 26 kDa band did in fact represent a dimeric form (Fig. 3d). This finding is consistent with descriptions of OsmC-like proteins that are dimeric in nature from various other bacteria (Lesniak et al., 2003; Rehse et al., 2004). The presence of dimeric forms in the recombinant protein preparations, but not the whole-cell extracts, suggests that different folding kinetics may occur during the process of protein purification.

Whole-cell proteins extracted from M. gallisepticum cells harvested at lag, and early, mid- and late-exponential, as well as stationary phase, were immunoblotted against MG1142 antiserum. The amount of protein expressed did not vary substantially throughout the growth cycle (data not shown), suggesting that MG1142 is likely to be constitutively expressed.

MG1142 resides on the cell surface

OsmC-like proteins are described in the literature as ‘putative envelope proteins’ (Davalos-Garcia et al., 2001; Sturny et al., 2003; Toesca et al., 2001). MG1142 belongs to the OsmC family of proteins; therefore, we speculated that it would be a good candidate for a cell-surface protein. In immunoblots of M. gallisepticum whole cells (Fig. 3e), MG1142 antiserum was shown to interact with the intact cells, indicating that MG1142 epitopes are surface accessible. Similar conclusions were drawn from immunogold labelling of whole M. gallisepticum cells, in which significantly more labelling was seen on the surface of the cells treated with anti-MG1142 antiserum (data not shown).

Bioinformatic analysis of MG1142

The MG1142 protein sequence was examined for two consensus motifs that are thought to be responsible for heparin binding: XBBBX and XBBBXXBX, where B corresponds to a basic residue and X represents a hydrophobic amino acid (Cardin & Weintraub, 1989). Neither of these motifs was found to occur in the MG1142 protein sequence; however, numerous heparin-binding proteins that lack these consensus sequences have been identified (Margalit et al., 1993); therefore, we proceeded to analyse this protein for heparin-binding activity.

Heparin-binding assays

As preliminary 2D ligand-blotting data indicated that MG1142 was a good candidate for a heparin-binding protein, we employed the purified recombinant protein in a number of heparin-binding assays. Ligand dot blots of the recombinant protein confirmed that it was able to react reproducibly with biotinylated heparin (Fig. 4a). Interestingly, protein that was denatured (by boiling) prior to spotting did not appear to bind heparin as strongly as the undenatured protein, suggesting that the protein

Fig. 4. Reaction of biotinylated heparin with recombinant MG1142. (a) Dot blot of undenatured and denatured MG1142 protein (0.5 μg per dot) reacted with biotinylated heparin (lane 1), or stained with amido black (lane 2). (b) Binding of biotinylated heparin to immobilized MG1142. Total binding (■), specific binding (▲) and non-specific binding (●) curves are shown. The total binding was determined using a standard microtitre plate assay, while the non-specific binding was determined in the presence of a 50-fold excess of unlabelled heparin. The specific binding curve was generated by subtracting the non-specific binding from the total binding. Error bars indicate SD for triplicate readings.
conformation plays a role in this interaction. While 2D electrophoresis is also expected to result in protein denaturation due to the presence of SDS, it is likely that at least partial renaturation of MG1142 occurs upon blotting, explaining the reactivity observed on 2D ligand blots.

The specificity of binding between MG1142 and heparin was determined via a microtitre plate assay, in which increasing concentrations of biotinylated heparin were added to immobilized MG1142 in microtitre-plate wells; we then used these assays to generate an affinity constant for the interaction. In each assay, MG1142-specific antisera verified that the recombinant protein bound efficiently to the microtitre wells (data not shown). Fig. 4(b) shows the total, specific and non-specific binding between MG1142 and heparin. The non-specific binding, determined by blocking specific binding sites on the MG1142 molecule with unlabelled heparin, was shown to be minimal. In contrast, the specific binding curve was both dose-dependent and saturable, with a dissociation constant of $10 \pm 1.8 \text{ nM}$, indicating a strong interaction. The affinity constants from replicate experiments were comparable.

**Inhibition of heparin binding with sulfated polysaccharides**

Competitive binding assays employing sulfated polysaccharides as inhibitors were used to assess the nature of the interaction between the recombinant MG1142 protein and biotinylated heparin. Binding of MG1142 to biotinylated heparin was significantly inhibited by heparin and fucoidan, but not by mucin or chondroitin sulfate A or B (Fig. 5).

**DISCUSSION**

Bacterial adherence and colonization are complex processes involving a large array of molecules that act in a coordinated fashion. Binding of bacterial cells to components of the host ECM is increasingly recognized as an essential step in the pathogenesis of many infectious microorganisms (Burnett *et al.*, 2006; Chen *et al.*, 1995; Dubreuil *et al.*, 2002; Hytonen *et al.*, 2001; Jenkins *et al.*, 2006; Joh *et al.*, 1999; Menozzi *et al.*, 1996). As obligate parasites, mycoplasmas are dependent on their hosts for many essential nutrients and growth factors, and therefore have an intrinsic requirement for efficient cytadherence and colonization strategies. Several cytadhesins of *M. gallisepticum* have been identified (Goh *et al.*, 1998; Papazisi *et al.*, 2002); however, interactions at the microbe–ECM interface are only now being investigated. A recent study by May *et al.* (2006), in which two fibronectin-binding proteins were identified, highlights the importance of bacterium–ECM interactions in *M. gallisepticum*. The ability of *M. gallisepticum* cells to bind heparin, and the partial disruption of interactions between this organism and MRC-5 cells in the presence of heparin, indicate that GAGs may be specifically involved in *M. gallisepticum* adherence. Although MRC-5 cells have been shown to be useful in the identification of adhesins of *M. gallisepticum*, future studies might consider the effects of GAGs and other ECM components on the ability of *M. gallisepticum* to bind to tissues or cell lines derived from avian sources.

Recent studies have demonstrated that low-passaged *M. gallisepticum* is capable of invading non-phagocytic (epithelial) cells (Winner *et al.*, 2000), and that this ability has been linked to systemic dissemination of the organism *in vivo* and the subsequent formation of multiple infection loci (Much *et al.*, 2002). The mechanisms by which *M. gallisepticum* invades host tissues have not been investigated, but in other organisms, binding to components of the ECM appears to play a central role. The heparin-binding haemagglutinin (HBHA) of *Mycobacterium tuberculosis* binds to heparan sulfate-containing proteoglycans on the surface of epithelial cells, and is essential for the extrapolmonary dissemination of this organism (Pethe *et al.*, 2001). HBHA-deficient mutants of *Mycob. tuberculosis* are still able to colonize the respiratory tissues, but do not disseminate. Interestingly, a similar phenomenon has been observed with *M. gallisepticum* $R_{low}$, although the molecules responsible for the invasive phenotype have not yet been identified in this organism (May *et al.*, 2006; Much *et al.*, 2002; Winner *et al.*, 2000). Binding to heparin or heparin analogues is directly implicated in the extrapolmonary dissemination of *Mycob. tuberculosis*, since antibodies that block the heparin-binding domain of HBHA inhibit dissemination; thus, heparin binding by *M. gallisepticum* may also play a significant role in the invasion of its host.

Another means by which pathogens can exploit the ability to bind heparin is by recruiting this compound to their cell
surfaces. Heparin has the ability to bind a diverse array of host components; therefore, recruiting heparin to the bacterial surface greatly increases the binding capabilities of the pathogen through bridging interactions. In Neisseria gonorrhoeae, heparin binding by the opacity factor protein (Opa) facilitates an interaction with vitronectin (Duensing & Putten, 1998), which in turn mediates internalization by Chinese hamster ovary cells (Duensing & van Putten, 1997). In addition, heparin has been shown to act as a molecular bridge between various pathogens and exogenous chemokines and cytokines, which would allow modulation of the host inflammatory and immune responses (Duensing et al., 1999).

The implications of heparin binding by M. gallisepticum require further investigation, but a strategy such as the one described above would be of particular importance for such organisms with minimal genomes. Mycoplasmas possess some of the smallest genomes of all bacteria, and therefore possess a limited repertoire of proteins with which to facilitate host–pathogen interactions. By exploiting heparin as a molecular bridge, an organism such as M. gallisepticum would be able to mediate interactions with a diverse array of host components, despite lacking specific receptors for these molecules (Duensing et al., 1999).

The 1- and 2D ligand blots performed in this study indicate that M. gallisepticum contains several putative heparin-binding proteins, including the OsmC homologue MG1142. Whole-cell immunoblotting and immunogold-labelling experiments demonstrate that MG1142 protein is surface accessible, a finding that is consistent with a role for MG1142 in adherence. A lack of linear consensus motifs in MG1142 indicates that the ability of this protein to bind heparin is likely conformational (Hileman et al., 1998; Margalit et al., 1993). Electrostatic interactions between the positively charged basic residues and the negatively charged sulfate groups of heparin (Cardin & Weintraub, 1989; Margalit et al., 1993) are implicated in binding, due to the pattern of inhibition observed in the competitive binding assays. Fucoidan, a branched and highly sulfated fucose polymer (Patankar et al., 1993) strongly inhibits heparin binding, while chondroitin sulfate, a less sulfated molecule with a similar iduronate backbone structure to that of heparin, fails to act as a competitive inhibitor. These observations highlight the importance of the sulfate groups rather than the polysaccharide backbone in heparin binding.

Known heparin-binding proteins such as clusterin, antithrombin and the mycobacterial heparin-binding haemagglutinin have dissociation constants ($K_d$) for heparin within the range of 0.3 nM to 4 μM (Pankhurst et al., 1998). The affinity of MG1142 for heparin was comparable at 10 ± 1.8 nM, indicating that this interaction is likely to be of physiological significance. MG1142 has an even stronger affinity for heparin than either of the recently characterized heparin-binding proteins of Mycoplasma hyopneumoniae, the cilium adhesin (P97) and P159, which have affinity constants in the micromolar range (Burnett et al., 2006; Jenkins et al., 2006).

Bacterial pathogens with the ability to bind components of the host ECM avail themselves of a diverse array of advantages, including an enhanced ability to adhere to host tissues, invade host cells and evade the host immune system. While the precise role of heparin binding by M. gallisepticum in vivo remains to be elucidated, it is likely to contribute significantly to the overall pathogenicity of this organism.

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