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Identification of Lipoprotein MslA as a Neoteric Virulence Factor of *Mycoplasma gallisepticum*▽

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Many lipoproteins are expressed on the surfaces of mycoplasmas, and some have been implicated as playing roles in pathogenesis. Family 2 lipoproteins of *Mycoplasma pneumoniae* have a conserved "mycoplasma lipoprotein X" central domain and a "mycoplasma lipoprotein 10" C-terminal domain and are differentially expressed in response to environmental conditions. Homologues of family 2 lipoproteins are *Mycoplasma* specific and include the lipoprotein of *Mycoplasma gallisepticum*, encoded by the MGA0674 gene. Comparative transcriptomic analysis of the *M. gallisepticum* live attenuated vaccine strain F and the virulent strain R_{low}, reported in this study, indicated that MGA0674 is one of several differentially expressed genes. The MGA0674-encoded lipoprotein is a proteolytically processed, immunogenic, TX-114 detergent-phase protein which appears to have antigenic divergence between field strains R_{low} and S6. We examined the virulence of an R_{low} ΔMGA0674 mutant (P1H9) *in vivo* and observed reduced recovery and attenuated virulence in the tracheas of experimentally infected chickens. The virulence of two additional R_{low} ΔMGA0674 mutants, 2162 and 2204, was assessed in a second *in vivo* virulence experiment. These mutants exhibited partial to complete attenuation *in vivo*, but recovery was observed more frequently. Since only *Mycoplasma* species harbor homologues of MGA0674, the gene product has been renamed "*Mycoplasma*-specific lipoprotein A" (MslA). Collectively, these data indicate that MslA is an immunogenic lipoprotein exhibiting reduced expression in an attenuated strain and plays a role in *M. gallisepticum* virulence.

Mycoplasma gallisepticum is a respiratory and reproductive tract pathogen of poultry, and disease results in major economic losses on commercial farms. *M. gallisepticum* can be transmitted via inhalation of aerosolized respiratory secretions and can also be spread vertically to the offspring of infected hens. Attachment of the bacterium to host respiratory epithelium results in inflammation, metaplasia, and loss of cilia. *M. gallisepticum* infection also places infected chickens at increased risk of developing a more severe, and potentially fatal, polymicrobial disease known as chronic respiratory disease (CRD) (18). Several live attenuated vaccines (LAV) have been generated to manage *M. gallisepticum* disease on chicken farms, but little is known about the genetic basis for their attenuation. We recently undertook an investigation of the genetic means by which the LAV strain F became avirulent by sequencing its complete genome and comparing it with the genome of the virulent strain R_{low}. Deletions and mutations in many genes were identified as significantly altering the coding sequence and may have an effect on virulence. We showed that a transposon knockout of the "hypothetical" gene MGA1107 in R_{low} (gene deleted in strain F) resulted in attenuation of the organism *in vivo*, indicating that this gene plays a role in the pathogenesis of *M. gallisepticum* (33).

Lipoproteins (LPs) reside on the surfaces of the cell wall-less mycoplasmas and are important factors in pathogenesis. Much research has been conducted on the large family of variable lipoprotein hemagglutinins (*vlhA*) of *M. gallisepticum*, which appear to function to elude and subvert the host immune system by undergoing antigenic variation. While similar in structure, VlhAs are antigenically distinct, and it is believed that a primary VlhA is predominantly expressed within a population of bacterial cells. As the host mounts a humoral response against the primary VlhA, bacterial cells expressing a secondary VlhA proliferate, and the host's immune system selects for and eliminates the population expressing the primary VlhA. This method of immune evasion is believed to be responsible for *M. gallisepticum*'s ability to establish chronic infections, leading to a greater opportunity for spread (24). The VlhAs have also been shown to be involved in cytadherence to chicken tracheal explants and in attachment to red blood cells, which may aid in dissemination of the bacterium *in vivo* (9). Other than the VlhAs, little work has been conducted on the role(s) of *M. gallisepticum* lipoproteins with respect to the virulence of this pathogen.

Many lipoproteins have been reported to be expressed in *M. gallisepticum*'s close relative *M. pneumoniae*, and these segregate into 6 different gene families based on sequence similarity (13). Lipoproteins of family 2 have the same domain structure, which includes a "mycoplasma lipoprotein X" central domain and a "mycoplasma lipoprotein 10" C-terminal domain. These domains are present only in *Mycoplasma* spp., but their functions have not yet been elucidated. Homologues include *M. gallisepticum* MGA0674, *M. synoviae* MS53_0285, -0329, and -0334, *M. hyopneumoniae* MHP362, -363, -366, -377, and -378,

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M. bovis membrane lipoprotein P81, *M. genitalium* MG185 and MG260, *M. agalactiae* MAG5030 (membrane lipoprotein P80), *M. arthritidis* MARTH832 and MARTH844, *M. fermentans* MBIO0368, *M. conjunctivae* MCJ000120, -000130, -000140, -003280, and -006070, and *M. pulmonis* MYPU0240. Expression of all family 2 lipoproteins in *M. pneumoniae* has been confirmed by mass spectrometry, with the exception of MPN098 (however, MPN098 is reverse transcription-PCR [RT-PCR] positive) (13). Hallamaa et al. (14) reported differential transcription of selected *M. pneumoniae* lipoproteins in contact with human lung epithelial cells as well as under oxidative or acidic stress. Only family 2 lipoproteins exhibited differential expression under all three conditions, with upregulation of MPN199 and MPN200 when in contact with A549 cells, downregulation of MPN152, -200, and -408 in response to H_2O_2, and downregulation of MPN152, -200, and -408 after acidic stress. Cecchini et al. (4) reported differential gene transcription of *M. gallisepticum* after attachment for 1 h to MRC-5 human lung fibroblast cells. It appears that MGA0674 was downregulated approximately 2.5-fold under these conditions. While the significance of these results regarding virulence remains unclear at this time, it has been reported that the *M. hyopneumoniae* homologue MHP378 is expressed at the protein level and can be detected by porcine convalescent-phase serum on two-dimensional (2D) immunoblots of TX-114 detergent-phase proteins (22). These data indicate that this lipoprotein is both expressed during infection and recognized by the host, suggesting a possible role in pathogenesis.

This report describes experiments pertaining to the differential transcription of MGA0674 between *M. gallisepticum* strains R_{low} and F, as well as the *in vivo* immunogenicity of the MGA0674 protein. Our findings led to the hypothesis that MGA0674 is involved in the virulence of *M. gallisepticum*. Three independent and isogenic mutants, with insertions in different locations in the MGA0674 gene, resulted in partial to complete attenuation of virulence and reduced recovery *in vivo*. This is the first elucidation of the role of an *M. pneumoniae* family 2 lipoprotein homologue in the virulence of a *Mycoplasma* species. Given the large number of *Mycoplasma* spp. which contain similar lipoproteins, these findings may have implications for mycoplasma virulence in general.

MATERIALS AND METHODS

Culture conditions. *M. gallisepticum* strains R_{low} (passage 18) and F (passage 21) were cultured at 37°C in Hayflick's complete medium until growth was at mid-log phase, as determined by acid shift of the growth medium from red to orange.

RNA extraction. Mid-log-phase cultures were placed on ice for 15 min, followed by pelleting via centrifugation at 10,000 × *g* for 15 min at 4°C, and the supernatant was discarded. Total RNA was extracted from pelleted cells by use of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA from each strain was treated with a DNA-Free kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The DNase-treated RNA concentration was determined based on the optical density (OD) at a 260-nm wavelength, and quality was assessed by comparing 16S and 23S rRNA bands in a 1% formaldehyde gel. RNA extractions were performed in triplicate, each on a separate day.

Microarray hybridization. The microarrays used in this work have been described by Cecchini et al. (4). Two samples from each of the three DNase-treated total RNA preparations (2 technical replicates from each of the 3 biological replicates; 50 μg of each sample) from each strain were reverse transcribed using an amino allyl cDNA labeling kit (Ambion) according to the manufacturer's instructions. Each cDNA sample was labeled with either the Cy3 or Cy5 fluorophore (Amersham Biosciences, Buckinghamshire, United Kingdom), and technical replicates were labeled with opposite dyes. Excess dye was removed using Nuc-Away spin columns provided in the kit. Samples were then mixed to include 1 technical replicate from each strain, with opposite Cy fluorophores. Labeled cDNA was resuspended in hybridization buffer (MWG Biotech), heated for 2 min at 95°C, cooled on ice for 3 min, and hybridized to the microarray slide at 42°C with shaking at 100 rpm overnight. The slides were then washed three times with decreasing concentrations of SSC buffer (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium acetate] with 0.1% sodium dodecyl sulfate [SDS], 1× SSC, and 0.1× SSC) and then centrifuged for 5 min at 500 rpm to remove excess hybridization and SSC buffer.

Microarray data acquisition and analysis. Microarray slides were scanned using a GenePix 4000B scanner (Axon Instruments, Sunnyvale, CA), and data were acquired with the GenePix 4.0 software package (Axon Instruments). Slides were scanned with a final adjustment to the photomultiplier tube gain so that a channel ratio of 1:1 was observed over the area comprising the array features and a maximum signal was obtained for 1 or 2 features. Median pixel intensity data values were used with local area background subtraction. All microarray data analyses were performed using programs from the TM4 software suite (28). Briefly, a low-level filter was applied to all features in which both channels had a median pixel intensity of <500. The data were then normalized using a Lowess multiple regression analysis and subjected to a flip-dye consistency check and cross-slide replicate analysis. Only features that passed all of the above criteria were used in downstream analyses. Statistical significance of features was assessed using a one-class *t* test with a Welch approximation for unequal group variances and a cutoff *P* value of <0.05. The false discovery rate was controlled by a standard Bonferroni correction, using permutation of the *P* value.

SDS-PAGE and immunoblots. For 1D immunoblots, strain R_{low} whole-cell lysates, harvested at different stages during the growth cycle, were separated using SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane as previously described (8). The membrane was then probed with convalescent-phase serum from a bird (435) previously infected with R_{low} (10). For 2D SDS-PAGE, first-dimension immobilized pH gradient (IPG) strips (170 mm, linear pH 3 to 10; Bio-Rad, Richmond, CA) were prepared for focusing by submersion in MSS sample buffer {5 M urea, 2 M thiourea, 0.8% carrier ampholytes 3 to 10, 2% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 2% (wt/vol) sulfobetaine 3-10, 2 mM tributyl phosphine (Bio-Rad)} overnight. Conditions used for the extraction of *M. gallisepticum* proteins with Triton X-114 were as described previously (36). *M. gallisepticum* proteins (250 μg) that partitioned into the Triton X-114 detergent phase were precipitated, diluted with sample buffer to a volume of 360 μl, and loaded onto preswollen IPG strips (8). Isoelectric focusing was performed with a Bio-Rad isoelectric focusing unit for 85 kV-h at 20°C. Proteins were reduced, alkylated, detergent exchanged, and then resolved in the second dimension by SDS-PAGE as described previously (6, 8). Gels were stained overnight in colloidal Coomassie blue G-250 as described previously (8). For 2D immunoblots, SDS-PAGE gels were prepared as described above, transferred to nitrocellulose membranes, and probed with convalescent-phase serum from chicken 435, previously infected with R_{low}, or from a chicken previously infected with strain S6 (12).

Postseparation analyses. (i) MALDI-TOF MS. Protein spots were excised from gels, digested with trypsin, and processed for matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (8). Spectra were acquired using either a PerSeptive Biosystems Voyager DE-STR (Framingham, MA) or TofSpec2E (Micromass, Manchester, United Kingdom) mass spectrometer. Both instruments were equipped with 337-nm nitrogen lasers. All spectra were obtained in reflectron/delayed-extraction mode, averaging 256 laser shots per sample. Two-point internal calibration of spectra was performed based upon internal porcine trypsin autolysis peptides (842.5 and 2,211.10 $[M + H]^+$ ions). A list of monoisotopic peaks corresponding to the mass of generated tryptic peptides was used to search a modified translated version of the *M. gallisepticum* genome (25). Successful identifications were based on the number of matching peptide masses and the percent sequence coverage afforded by those matches. Mascot scores of >41 were considered significant ($P < 0.05$). N-terminal Edman sequencing was performed using a Procise 494 protein sequencer (PE Biosystems) as described previously (3).

(ii) LC-MS/MS. Using an Eksigent AS-1 autosampler connected to a Tempo nano-liquid chromatography (nano-LC) system (Eksigent), 10 μl of the sample was loaded at 20 μl/min with MS buffer A (2% acetonitrile plus 0.2% formic acid) onto a C_8 trap column (Michrom). After washing of the trap for 3 min, the peptides were washed from the trap at 300 nl/min onto an IntegraFrit column (75 μm × 100 mm) packed with ProteoPep II C_{18} resin (New Objective, Woburn,

TABLE 1. Differentially expressed genes between strains R_{low} and F

Gene product	Locus tag	R_{low}/F expression ratio[a]	*P* value
PvpA (putative variable cytadhesin protein)	MGA0256	12	0.003
Adenosine triphosphatase (AtpB)	MGA1164	8	0.048
VlhA3.03	MGA0383	8	0.048
Unique hypothetical protein	MGA0482	6	0.046
Conserved hypothetical protein	MGA0674	6	0.025
Cytosol aminopeptidase	MGA0114	4	0.008
Dipeptide transport system; permease protein (DppB)	MGA0224	3.5	0.039
Hypothetical protein	MGA0867	3.5	0.007
Purine-nucleoside phosphorylase	MGA0364	3	0.010
Conserved hypothetical protein	MGA0035	−12	0.014
NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	MGA0860	−4	0.025
Elongation factor Tu (EF-Tu)	MGA1033	−3.5	0.040
50S ribosomal protein L11	MGA0503	−3.5	0.022
Conserved hypothetical protein (lipoprotein)	MGA0565	−3	0.010

[a] A positive ratio indicates a higher expression level in R_{low}, whereas a negative ratio indicates a higher expression level in the F strain. Data were rounded to the nearest half-fold change.

MA). Peptides were eluted from the column and into the source of a QSTAR Elite hybrid quadrupole–time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex), using the following program: 5 to 50% MS buffer B (98% acetonitrile plus 0.2% formic acid) over 15 min, 50 to 80% MS buffer B over 5 min, 80% MS buffer B for 2 min, and 80 to 5% MS buffer B over 3 min. The eluting peptides were ionized with a 75-μm-internal-diameter emitter tip that tapered to 15 μm (New Objective) at 2,300 V. An intelligent data acquisition (IDA) experiment was performed, with a mass range of 375 to 1,500 Da continuously scanned for peptides with a charge state of 2+ to 5+, with an intensity of >30 counts/s. Selected peptides were fragmented and the product ion fragment masses measured over a mass range of 100 to 1,500 Da. The mass of the precursor peptide was then excluded for 15 s.

Mass spectrometry data analysis. The MS/MS data files produced by the QSTAR machine were searched using Mascot Daemon (version 2.2.2; provided by the Australian Proteomics Computational Facility [http://www.apcf.edu.au/]) against the LudwigNR database (comprised of the UniProt, plasmoDB, and Ensembl databases [vQ209; 8,785,680 sequences and 3,087,386,706 residues] [www.apcf.edu.au]), with the following parameter settings: fixed modifications, none; variable modifications, propionamide, oxidized methionine; enzyme, trypsin; number of allowed missed cleavages, 3; peptide mass tolerance, 100 ppm; MS/MS mass tolerance, 0.2 Da; and charge state, 2+ and 3+. The results of the search were then filtered by including only protein hits with at least one unique peptide and excluding peptide hits with *P* values of >0.05. Peptides identified by Mascot were further validated by manual inspection of the MS/MS spectra for the peptide to ensure that the *b* and *y* ion series were sufficiently extensive for an accurate identification (data not shown).

***M. gallisepticum* R_{low} transformation and mutant identification.** Mutant P1H9 was previously generated by our lab, as described by Hudson et al. (15). A library of 3,600 R_{low} transposon mutants was generated via electroporation (Gene Pulser; Bio-Rad, Hercules, CA) as described by Hudson et al. (15), using the plasmid pMT85 (a gift from Kevin Dybvig). This plasmid carries the transposon mini-Tn*4001*-gent (encodes an exogenous transposase), which harbors a gentamicin resistance gene. Upon growth of clonal mutants (grown in 200 μg/ml gentamicin), the culture was split into individual 1-ml aliquots (stored at −80°C), and a 500-μl aliquot of each clone was added to a pool with 29 other mutants which exhibited similar growth rates (total of 30 mutants/pool). Pools were grown in 300 ml of Hayflick's medium at 37°C until a color shift from red to orange was apparent. Genomic DNAs were extracted from the pool by using an Easy-DNA extraction kit (Invitrogen, Carlsbad, CA) per the manufacturer's instructions.

PCR screening of mutant pools for transposon insertions in the MGA0674 gene was performed in 96-well plates by using a gene-of-interest-specific forward (5′) primer (SG2188 [TGATTGAACGCGAAGTT]) in conjunction with a transposon-specific primer (SG1992 [ATGAGTGAGCTAACTCACAG] or SG1993 [CAATACGCAAACCGCCTC]) directed from the 5′ or 3′ end of the transposon. PCR was performed on positive pools by using a gene-of-interest-specific reverse (3′) primer (SG2189 [AGCCACCCTTGTTTTTTCGAA]) and the corresponding transposon-specific primer, depending on the orientation of the transposon. Pools which had positive reverse PCRs and for which the forward and reverse amplicon sizes added to the appropriate total were selected for a second round of screening of individual mutants. DNAs for PCR screening of individual mutants were extracted using Chelex reagent per the manufacturer's instructions, and PCR was conducted in the same manner as that described for the mutant pools.

The transposon insertion site was identified as described by Hudson et al. (15). Mutants were considered isogenic if the sequencing chromatograph contained only 1 major trace arising from a single transposon insertion.

Animals. Four-week-old female specific-pathogen-free White Leghorn chickens (SPAFAS, North Franklin, CT) were used. Upon arrival, the birds were divided into groups of six, tagged, placed in HEPA-filtered isolators (Controlled Isolator Systems, Pittsburgh, PA), and allowed to acclimate for 1 week prior to experimentation. Nonmedicated feed (Blue Seal, Waltham, MA) and water were provided *ad libitum* throughout the experimental period. The chicken study procedures described in this work were conducted in accordance with state and federal policies to ensure the humane use and care of the research animals. The protocol has been approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC) (protocol A07-001).

Chicken studies. Cultures of mutants P1H9, 2162, and 2204 and of wild-type R_{low} were grown from frozen stocks at 37°C with shaking at 130 rpm in fresh Hayflick's medium for 5 h prior to animal inoculation. The *Mycoplasma* concentration was determined using OD_{620} measurements and 10-fold serial dilutions (to measure color-changing units [CCU]) to ensure that the *M. gallisepticum* cells measured were alive. Chickens were challenged intratracheally with 1×10^8 CFU/200 μl of the respective mutant or strain or with Hayflick's medium alone on days 0 and 2. At 2 weeks postinoculation, chickens were euthanized and immediately necropsied. Gross and histopathological examinations of lesions and recovery of mycoplasmas (as measured by CCU) were conducted as described by Gates et al. (10).

Statistical analysis. Tracheal measurements and lesion scores were assessed using the nonparametric Kruskal-Wallis analysis of variance on ranks test, in which all post hoc pairwise multiple comparison procedures were performed using Dunn's method (for groups of unequal size) or the Student-Newman-Keuls method (for groups of equal size). A *P* value cutoff of <0.05 was used to determine statistical significance. All data were analyzed using SigmaPlot v. 11 (Jandel Scientific, San Rafael, CA).

RESULTS

Microarray comparison of the transcriptomes of strains R_{low} and F. Genomic comparisons of *M. gallisepticum* strains R_{low} and F were performed as a means of understanding genetic changes which may account for their differences in virulence in chickens (33). Following that study, oligonucleotide DNA microarrays were used in a functional genomic approach to compare the *in vitro* transcriptomes of these strains to identify additional differences which may explain their observed virulent and attenuated phenotypes. A total of nine genes were

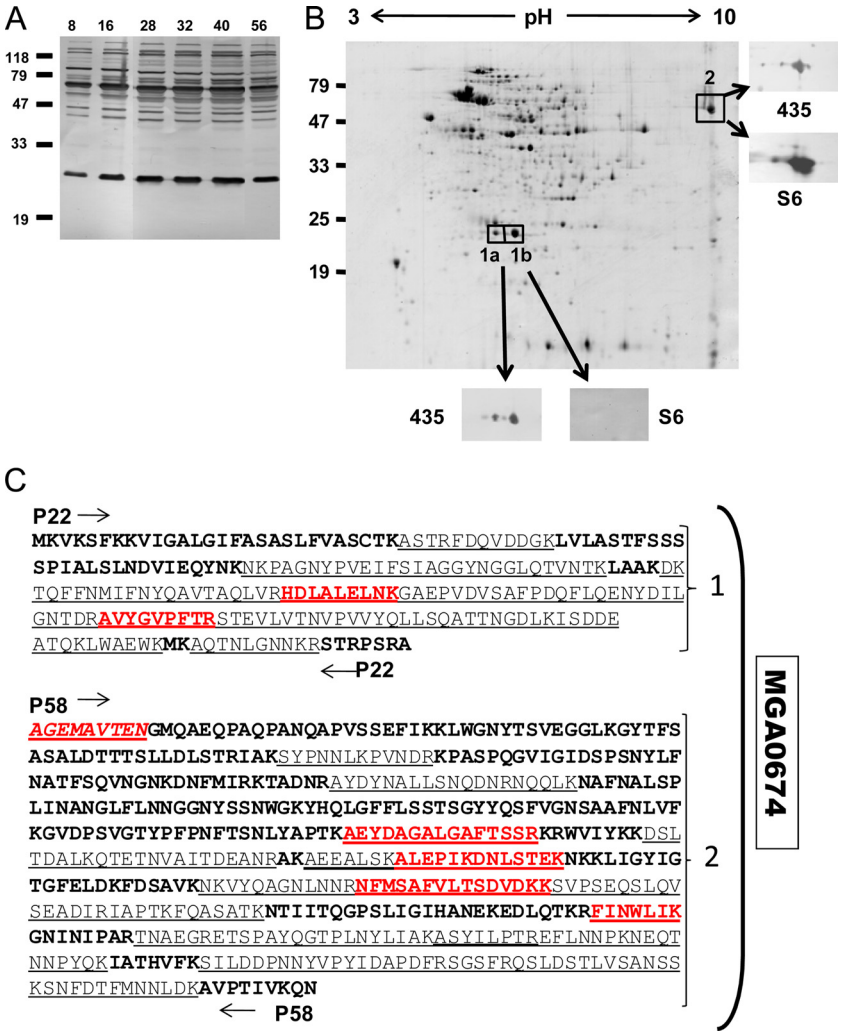

FIG. 1. P22 and P57 are cleavage fragments of MGA0674. (A) R_{low} whole-cell lysates harvested at different stages during the growth cycle and probed with convalescent-phase serum from bird 435. (B) R_{low} proteins were fractionated with Triton X-114, and detergent-soluble proteins were separated by 2D PAGE and stained with colloidal Coomassie blue. Proteins were initially resolved in a pH gradient of 3 to 10, followed by SDS-PAGE through a 16% polyacrylamide gel. The protein in spot 2 reacted with convalescent-phase chicken sera raised against R_{low} (bird 435) and S6. Proteins 1a and 1b reacted with convalescent-phase serum raised against R_{low} (bird 435) but not with serum raised against strain S6. (C) MALDI-TOF MS and LC-MS/MS analyses were used to characterize tryptic digests of protein spots in boxes. MALDI-TOF MS analyses of tryptic digests of spots 1a and 1b mapped to the N-terminal 22-kDa cleavage fragment (P22) of MGA0674. Spot 2 mapped to the C-terminal 57-kDa cleavage fragment (P57) of MGA0674. Tryptic peptides identified by MALDI-TOF MS are underlined. Seven peptides in total were identified by LC-MS/MS (shown in red). Two of these came from spot 1b (P22) and had the sequences HDLALELNK (peptide score, 79) and AVYGVPFTR (peptide score, 50). The remaining five peptides localized to the protein found in spot 2 (P57). These five peptides had the following sequences: AEYDAGALGAFTSSR (peptide score, 92), ALEPIKDNLSTEK (peptide score, 77), NFMSAFVLTSDVDKK (peptide score, 61), FINWLIK (peptide score, 37), and ASYILPTR (peptide score, 47). N-terminal sequence analysis of the protein in spot 2 produced the sequence AGEMAVTEN (shown in red italics) and identified the precise cleavage site in MGA0674, at alanine 224/alanine 225.

identified as having greater expression in R_{low}, while five had greater expression in strain F (Table 1). MGA0674 exhibited 6-fold higher expression in R_{low} than in strain F.

Gel electrophoresis and protein identification. Convalescent-phase serum from R_{low}-infected bird 435 consistently recognized R_{low} proteins with approximate masses of 24, 44, 46, 48, 58, 66, 72, 74, and 85 kDa and several proteins with masses of >120 kDa at different stages (8 to 48 h) of the growth cycle (Fig. 1A). As part of a larger study to identify these proteins, R_{low} proteins were fractionated using TX-114. Most immunodominant antigens recognized by bird 435's serum partitioned to the TX-114-soluble phase and were densely clustered between pIs 4 and 7 (data not shown), with masses ranging from 40 to 110 kDa. Immunoreactive proteins, identified as spots 1a, 1b, and 2 in the TX-114 detergent fraction (Fig. 1B), were unusual because they resolved to regions on the blot that were clear of other immunoreactive proteins, and they were characterized further. Furthermore, a blot of TX-114 detergent-soluble R_{low} proteins incubated with convalescent-phase chicken serum raised against strain S6 reacted with the C-terminal (spot 2) but not the N-terminal (spots 1a and 1b) fragments of MGA0674 (Fig. 1B). MALDI-TOF MS analyses

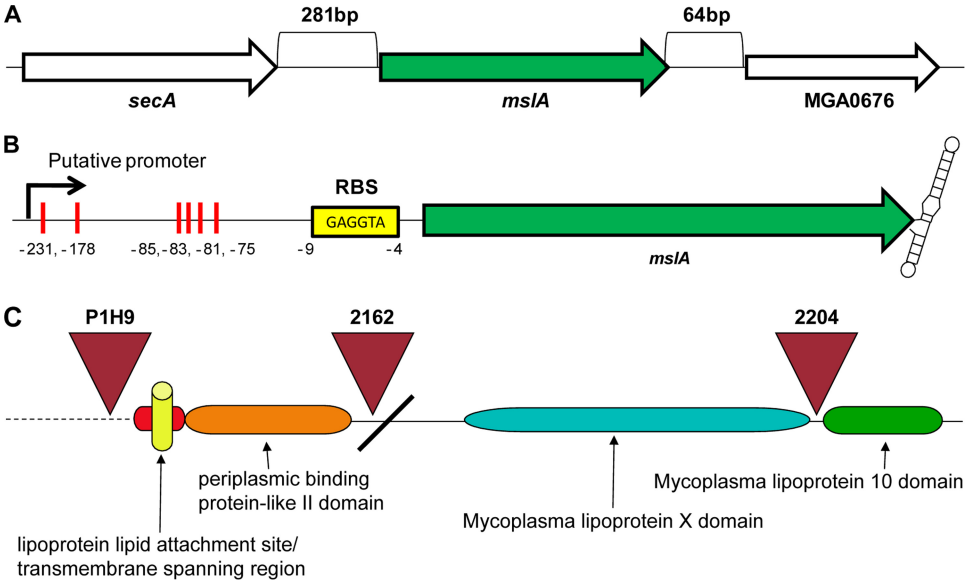

FIG. 2. *In silico* analysis of the *mslA* gene. (A) Genomic architecture of the *mslA* locus. Open reading frames (ORFs) are indicated with arrows, and the distances (bp) from *mslA*-flanking genes are indicated by brackets. (B) Genetic organization of *mslA*. The 281-bp upstream sequence is considered the putative promoter region, and SNPs in this region between strains R_{low} and F are indicated by red vertical lines. The putative RBS is shown as a yellow box, and the ORF is represented with a green arrow. The Rho-independent transcriptional terminator is shown as the tandem hairpin structure 3′ of the ORF. (C) Domain structure and transposon mutant insertion locations. The regulatory sequence is indicated by a dashed line, transposon insertion sites are represented by inverse triangles, the transmembrane spanning region is indicated by a cylinder, and the P22/P57 cleavage site is shown as a diagonal slash.

of tryptic fragments generated from proteins found in spots 1a and 1b and in spot 2 showed that they contained N- and C-terminal fragments of MGA0674, respectively (Fig. 1C). Two spots (1a and 1b) migrated with a mass of approximately 22 kDa (P22) and pI 6 to 6.5. MALDI-TOF MS analysis of spot 1b identified 8 tryptic peptides (17% sequence coverage) within the N-terminal 199 amino acids of MGA0674 (Mascot score, 84), and analysis of spot 1a identified 10 peptides (19% coverage), also within the N terminus of MGA0674 (Mascot score, 89). Spot 2 migrated with a mass of approximately 57 kDa (P57) and a pI of ~9. MALDI-TOF MS analysis of spot 2 identified 12 tryptic peptides (21% sequence coverage) that mapped within the C-terminal 522 amino acids of MGA0674 (Mascot score, 146). N-terminal sequence analysis of the protein eluted from spot 2 generated the sequence AGEMAVTEN, confirming that the C-terminal fragment of MGA0674 commenced at alanine 225 and spanned amino acids 225 to 721. These migration parameters are consistent with the predicted mass and pI of each cleavage fragment determined by ProtParam (ExPASy Proteomics Server [http://ca.expasy.org/tools/protparam.html]).

***In silico* analysis and renaming of MGA0674 to *Mycoplasma*-specific lipoprotein A (*mslA*).** The genetic and structural details of MGA0674 are outlined in Fig. 2. Domain analysis was conducted using the program InterProScan (26), and transmembrane spanning regions were identified using the dense alignment surface method (7). MGA0674 has a typical prokaryotic lipoprotein lipid attachment site domain which also contains a hydrophobic membrane-spanning region. A lipoprotein signal sequence and a lipid attachment site were found using the hidden Markov model (HMM) of the LipoP 1.0 program (17), which identified a Ser-25/Cys-26 protease cleavage site. This leaves the essential cysteine available for attachment of a lipid moiety, as is commonly found in other bacteria. These *in silico* analyses correlate with the observation that MslA fractionates into the TX-114 detergent phase, which constitutes mainly membrane proteins. Immediately downstream of the signal sequence is a periplasmic binding protein-like domain, but since mycoplasmas do not have a periplasm, the significance of this finding is unclear. The P22/P57 cleavage site is marked with a slash in Fig. 2. Downstream, a central "mycoplasma lipoprotein X" domain and a "mycoplasma lipoprotein 10" domain are present, exclusively in P57.

As previously mentioned, the functions of the above domains are currently unknown, but they are found only in members of the genus *Mycoplasma*. This prompted the renaming of MGA0674 to "*Mycoplasma*-specific lipoprotein A" (*mslA*). Only one copy of the gene has been identified in *M. gallisepticum*, whereas there are many homologues in numerous other *Mycoplasma* species.

Genomic analysis of R_{low} (25) by use of the program Artemis v. 10 (27) indicated that *mslA* is sufficiently distant from other genes (281 bp in the 5′ putative promoter region and 64 bp in the 3′ region) that it is not likely a member of an operon (Fig. 2). Downstream of the translational stop codon, a tandem hairpin secondary RNA structure was predicted using the program GeneBee (2). A predicted ribosome binding site (RBS) (GAGGTA) was identified starting 4 bp upstream of the translational start site (TSS), using the program Metagene (35). Sequencher (Ann Arbor, MI)-based multiple sequence alignments of the putative promoter region of *mslA* identified single nucleotide polymorphisms (SNPs) between R_{low} and the F strain at positions 74, 81, 83, 85, 178, and 231 bp upstream of

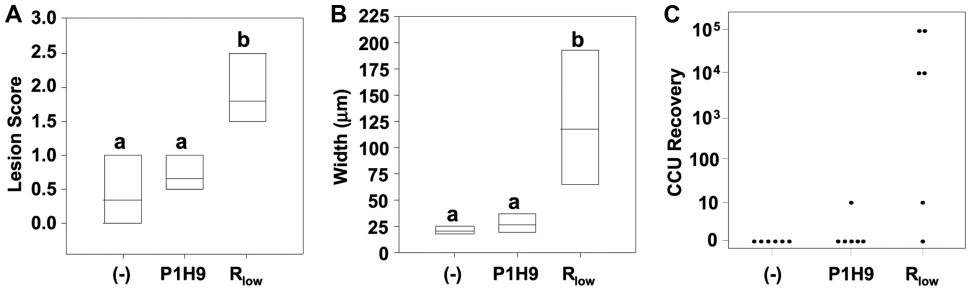

FIG. 3. Results of *in vivo* virulence assessment study 1. Tracheal lesion scores (A) and tracheal mucosal thicknesses (B) were determined for chickens inoculated with strain P1H9 or R_{low} of *M. gallisepticum* or with Hayflick's medium alone. (−), medium-inoculated group (negative control). The 25th percentiles, medians, and 75th percentiles are represented by box plots, and letters indicate statistically similar groups, using Dunn's or the Student-Newman-Keuls method. (C) Recovery of *M. gallisepticum* from chicken tracheas. The *y* axis indicates the maximum number of cells recovered given the highest serial dilution exhibiting a color shift for a given chicken.

the TSS, which may explain the differences in transcription observed in the microarray experiments.

Since the cleaved P57 fragment of MslA is no longer attached to a lipid anchor, it was unexpected to find it in the detergent fraction of 2D gels. The dense alignment surface method did not yield any obvious transmembrane spanning regions; however, the TopPred (5) and PSIPRED (21) programs identified a hydrophobic region with limited alpha-helical secondary structure (spanning amino acids 391 through 409) which may span the membrane. TopPred predicts the N terminus of P57 to be extracellular, while the C terminus appears to be cytoplasmic. This prediction correlates with intact MslA expression on the cell surface followed by cleavage into two products, yielding an extracellular N terminus for P57. Based on this prediction, the mycoplasma lipoprotein X and mycoplasma lipoprotein 10 domains should be cytosolic and not accessible for interaction with host cells.

MslA was found to be immunogenic, and the presence of continuous linear B-cell epitopes was predicted by the SCRATCH protein predictor (32) for both P22 and P57; these included DDEATQK (starting at amino acid 192; 83% probability), KASTRF (starting at amino acid 27; 82% probability), and KGAEPVD (starting at amino acid 127; 81% probability) for P22 and VSSEFIK (starting at amino acid 248; 76% probability), GGLKGY (starting at amino acid 265; 76% probability), and GNYTSV (starting at amino acid 258; 73% probability) at the N terminus of P57. It is interesting that the 3 top hits for B-cell epitopes are found at three different locations within P22, while they are clustered together in P57. These predictions indicate that there may be multiple immunogenic sites on P22 but only one major immunogenic site for P57.

The transposon insertion site for each mutant was determined by genomic DNA sequencing, utilizing outward-directed primers anchored within the transposon (Fig. 2). Sequence analysis was performed using the Sequencher program. Mutant P1H9 was determined to have an insertion 7 bp upstream of the TSS, thereby interrupting the predicted RBS. Mutant 2162 has an insertion 638 bp into the gene, which is 34 bp upstream of the P22/P57 protease cleavage site. The transposon in mutant 2204 is inserted 1,667 bp into the gene, which is between the mycoplasma lipoprotein X and mycoplasma lipoprotein 10 domains.

***In vivo* virulence assessment of transposon mutants. (i) Experiment 1.** The virulence of *mslA* mutant P1H9 was assessed in White Leghorn chickens ($n = 6$ per group). Air sac and lung lesions have historically been shown to be too variable to draw statistically significant data from, so for the sake of consistency and reproducibility, the analysis presented in this study was performed with tracheal lesions, as described previously (10, 15). Histopathologic lesions, mucosal thickness, and recovery of *M. gallisepticum* mutant P1H9 from tracheas were compared with those for both R_{low}-infected birds and control birds, which received *Mycoplasma* growth medium only (Fig. 3). Microscopic lesion scores of P1H9-inoculated birds (median = 0.75) were not statistically different from those of control chickens (median = 0) but were statistically different from those of R_{low}-infected birds (median = 1.75) (all data were significant, with P values of <0.05, unless otherwise noted). Measurements of the thicknesses of tracheal mucosae also showed that P1H9-inoculated chickens were similar to control birds but different from those inoculated with R_{low}. No mycoplasmas were recovered from the control birds, but one of six P1H9-inoculated chickens and five of six R_{low}-inoculated chickens were culture positive.

(ii) Experiment 2. Complementation of the P1H9 mutant was unsuccessful (no complemented mutants generated), consistent with a previous report by Hudson et al. (15), so two additional *mslA* mutants, 2162 and 2204, were assessed to determine if they also resulted in the avirulent phenotype. Mutants P1H9, 2162, and 2204, along with growth medium and R_{low}, were each inoculated into different groups of chickens in the same manner as in the previous study (Fig. 4). Microscopic lesions of P1H9-inoculated birds were identical to those observed in growth medium-inoculated chickens (median lesion score = 0.5 for both groups) and were statistically different from those induced by R_{low} (median lesion score = 1.75). Lesions for mutant 2162 (median = 0.75) were not statistically different from those induced by growth medium but were statistically different from those induced by R_{low}. Mutant 2204 caused lesions (median lesion score = 1.0) that were not statistically different from those induced by R_{low}. However, using Grubb's test (outlier analysis), it was determined that a lesion score of 2.5 for 1 bird skewed the data for this group ($P < 0.01$). Analysis excluding this datum point resulted in an inter-

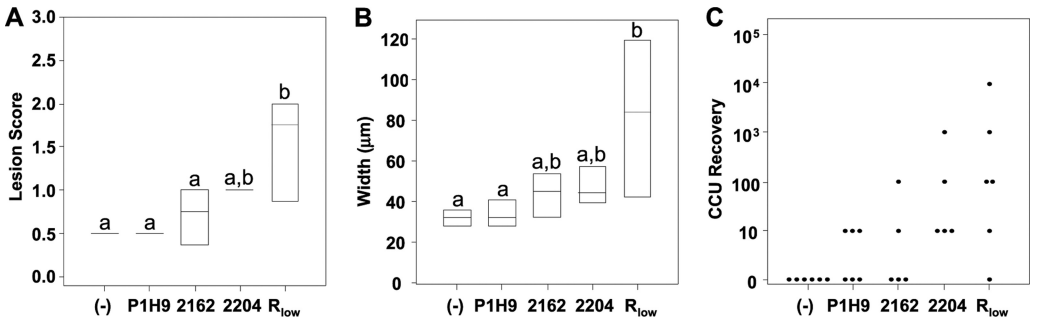

FIG. 4. Results of *in vivo* virulence assessment study 2. Tracheal lesion scores (A) and tracheal mucosal thicknesses (B) were determined for chickens inoculated with strain P1H9, 2162, 2204, or R_{low} of *M. gallisepticum* or with Hayflick's medium alone. (−), medium-inoculated group (negative control). The 25th percentiles, medians, and 75th percentiles are represented by box plots, and letters indicate statistically similar groups, using Dunn's or the Student-Newman-Keuls method. (C) Recovery of *M. gallisepticum* from chicken tracheas. The *y* axis indicates the maximum number of cells recovered given the highest serial dilution exhibiting a color shift for a given chicken. (Note that data are not presented for 1 bird from the mutant 2204-inoculated group that was determined to be a statistical outlier, nor are recovery data presented for 1 bird from the mutant 2164-inoculated group that was determined to be contaminated.)

mediate phenotype which was not statistically different from those observed with either growth medium or R_{low} (which were statistically different from each other). Tracheal thickness measurements for the P1H9 and growth medium groups were not statistically different, while results for mutants 2162 and 2204 were not statistically different from those for either growth medium or R_{low} (which were statistically different from each other). *M. gallisepticum* was not recovered from the tracheas of control birds, but it was recovered from three P1H9-inoculated chickens, two of five mutant 2162-inoculated birds (1 culture was eliminated from the analysis due to contamination), and five of five mutant 2204-inoculated birds. *M. gallisepticum* was recoverable from five of six R_{low}-inoculated chickens.

DISCUSSION

It is well established that lipoproteins play roles in the pathogenesis of mycoplasmal diseases. Barring lipoproteins involved in attachment, experimental evidence that lipoproteins are in fact virulence factors and are directly involved in the development of lesions in experimental animals is sparse. In this study, three independent isogenic mutants with mutations in the previously uncharacterized *mslA* gene of *M. gallisepticum* were shown to be attenuated in the tracheas of inoculated chickens. These findings carry broader significance for the field of mycoplasmology, as other mycoplasmas which harbor homologues to *mslA* may also be attenuated by disruption or deletion of these genes.

It was previously demonstrated by microarray analysis that *M. gallisepticum* differentially transcribes genes after 1 h of incubation with eukaryotic cells versus growth in medium alone (4). These conditions would not allow for outgrowth of subpopulations, as the generation time of *M. gallisepticum* is approximately 2 h. More recently, a two-component signal transduction system was putatively identified in the *M. penetrans* genome (16), and seven previously unidentified transcription factors have been characterized empirically in *M. pneumoniae* (38), which contributes more evidence that mycoplasmas can sense and respond to the milieu in which they reside. In this study, genomic comparisons of strains R_{low} and F were undertaken at the functional level, utilizing microarrays described in a previous transcriptomic study. Among the differentially transcribed genes identified, *mslA* showed a 6-fold reduction of expression in the attenuated F strain. R_{low} and F strain sequence comparisons of the predicted promoter region of this gene revealed SNPs at positions 74, 81, 83, 85, 178, and 231 bp upstream of the TSS. Differential expression of the *vlhA* genes of *M. gallisepticum* is reported to be induced by variations of the number of GAA trinucleotide repeats, which begin 50 bp upstream of the gene TSS (19), indicating that *M. gallisepticum* promoters can be very large and that the SNPs observed in the *mslA* promoter region likely result in the reduced transcription observed in strain F. Previously, it was observed that the "conserved hypothetical" gene MGA1107 was upregulated approximately 3.5 times when R_{low} was attached to MRC-5 cells versus growth in culture medium, and it was shown to be missing from the F strain genome by sequencing (4, 33). It has also been reported that a transposon mutant of MGA1107 resulted in attenuation of R_{low} in chickens. The reduced expression of *mslA* in the F strain and the attenuation of R_{low} *mslA* mutants *in vivo* are consistent with the results observed for MGA1107. These results demonstrate the utility of genomic comparisons as a means of identifying factors involved in virulence. This also indicates that the reduced virulence of vaccine strains is likely multifactorial in nature.

Identification of immunogenic surface proteins is a useful means of identifying factors that are involved in host-pathogen interactions. By utilizing 2D gel electrophoresis with mass spectrometry, two spots within the detergent (hydrophobic) fraction of strain R_{low} following TX-114 partitioning were identified as MslA. N-terminal sequencing of P57 identified the protease cleavage site of the protein portion of the mature LP. 2D immunoblotting of the same protein fraction with chicken anti-R_{low} antiserum revealed that both P22 and P57 are immunogenic, but only P57 was recognized by anti-S6 antiserum. These data indicate that MslA is recognized by the host during infection and that there may be strain differences in its structure which may have an effect on the host's ability to recognize and mount a response against this lipoprotein. As mentioned above, the *vlhA* genes are thought to create anti-

genic diversity within the *M. gallisepticum* population which permits the bacterium to evade the host's humoral immune response and allow for the establishment of chronic infections (24). It is plausible that the strain differences in immunoreactivity of MslA provide a similar mechanism of immune evasion (at the species level) which allows *M. gallisepticum* to persist on farms. Many of the remaining R_{low} detergent-phase proteins were not recognized by anti-S6 convalescent-phase sera, but P57 of MslA was. This indicates that P57 may be conserved across strains and could be a useful diagnostic marker for *M. gallisepticum* infection in flocks of birds. Also, the linear B-cell epitopes predicted in P22 were found in very different locations of the lipoprotein, but the epitopes of P57 were tightly clustered together. It is possible that P57 maintains a large region that is highly immunogenic and is thus more conserved across strains.

MGA0674 transposon mutants were tested *in vivo* to determine if MslA plays a role in the virulence of *M. gallisepticum*. *In vivo* experiment 1 was designed to assess the virulence of mutant P1H9. This mutant produced less-severe lesions in the tracheas of inoculated chickens and had reduced recovery compared to R_{low}. Given these results, two additional mutants with transposon insertions in the *mslA* gene were selected and assessed for their effect on virulence. Mutant 2162 contains an insertion 34 bp before the P22/P57 cleavage site, and mutant 2204 contains an insertion between the mycoplasma lipoprotein X and mycoplasma lipoprotein 10 domains. In the second *in vivo* virulence experiment, mutants P1H9 and 2162 were completely attenuated, while mutant 2204 was partially attenuated. This indicates that a knockout of the putative RBS, P57 fragment, or mycoplasma lipoprotein 10 domain results in attenuation of virulence. Given these results, no portion of the gene appears to be dispensable for virulence of the organism. Much work has been done recently to characterize *mslA* homologues in other mycoplasmas (13, 14, 22), but a definitive link to virulence has not been established. The results of the *in vivo* infection studies from this work indicate that MslA is a virulence factor of *M. gallisepticum* and raise the question of whether other members of this family of lipoproteins play roles in the virulence of other mycoplasmas.

While the data presented in this work indicate that MslA is an important factor in the pathogenesis of *M. gallisepticum*, it is still not known what function(s) it may play. In other bacteria, much emphasis has been placed on the role of the lipid region of lipoproteins in the activation of Toll-like receptors (TLRs) of the host's innate immune system. Diacylated lipoproteins of mycoplasmas bind to preformed TLR2/6 heterodimers, whereas triacylated lipoproteins bind to preformed TLR2/1 heterodimers. Both are trafficked to CD14-containing lipid rafts on the host cell membrane (TLR2/6 also requires CD36 in the rafts), at which point intracellular TLR signaling and raft complex targeting to the Golgi apparatus take place (34). Downstream NF-κB activation is therefore the same with diacylated and triacylated lipoproteins, but downstream effects may differ depending on the structure of the fatty acids incorporated into the lipid moiety or the amino acid sequence of the protein (30). Mycoplasmal lipoprotein-induced NF-κB expression has been linked to the production of cytokines and chemokines, which can be detected both *in vitro* and *in vivo* and results in inflammation in experimental animals (20, 31). Another downstream effect of NF-κB activation is the induction of apoptosis of affected cells, and mycoplasmal lipoproteins have been shown to induce this form of cell death *in vitro* (37, 39). However, Gerlic et al. (11) have shown that *M. fermentans* total lipoproteins and the synthetic lipopeptide MALP-2 have an antiapoptotic effect on U937 cells. It has been suggested that mycoplasmal lipoproteins are capable of both induction and inhibition of apoptosis, which may be important for transitioning to different phases of infection. Although this remains to be determined experimentally, it is likely that MslA binds TLRs and activates NF-κB, as these are common consequences of lipoprotein contact with host cells. Which cytokines are up- or downregulated and whether apoptosis is induced or inhibited in response to MslA are more complex questions that can be answered only with additional experimentation.

It is interesting to observe the effects that mycoplasmal lipoproteins have on host cells, but it is likely that they also serve other functions for the bacterium. The *M. arthritidis* lipoprotein Maa1 has been shown to be essential for cytadherence to rat lung cells, as both natural and transposon mutants show reduced attachment compared to the wild-type strain. In contrast, the lipoprotein Maa2 appears to be suppressive or modulatory in its role in cytadherence, as a transposon mutant of this gene resulted in increased attachment (1). Mycoplasmal lipoproteins also serve other functions. For example, the *M. hyopneumoniae* lipoprotein MHP379 has functional activity as an exonuclease (29), and *M. penetrans* lipoproteins P38 and P35 are both capable of binding to human secretory IgA (23). While experiments with these lipoproteins have only been performed *in vitro*, their functions suggest that they play an important role in the survival of these pathogens in the host.

In summary, we have demonstrated that MslA is an immunogenic lipoprotein that exhibits reduced expression in a vaccine strain and that *mslA* knockout mutants attenuate R_{low} virulence *in vivo*.

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