Molecular and biochemical analysis of a 105 kDa Mycoplasma gallisepticum cytadhesin (GapA)

M. S. Goh, T. S. Gorton, M. H. Forsyth,† K. E. Troy and S. J. Geary

The identification of a gene (gapA) from Mycoplasma gallisepticum with homology to the P1 cytadherence gene of Mycoplasma pneumoniae is reported. The gapA gene is a 2895 bp ORF encoding a protein with a molecular mass of 105 kDa. Nucleotide sequence analysis of the gapA gene revealed 45% homology to the M. pneumoniae P1 gene, 46% homology to the Mycoplasma genitalium MgPa gene and 47% homology to the Mycoplasma pirum P1-like protein gene. It has a 64 mol% A+T content compared to 46, 60 and 72 mol% respectively for the P1, MgPa and the P1-like protein genes. As with the P1 and MgPa genes, gapA is a central gene in a multi-gene operon, but unlike the P1 and MgPa genes, there is only a single copy of gapA in the genome. GapA is a trypsin-sensitive surface-exposed protein. Chicken tracheal-ring inhibition-of-attachment assays, using anti-GapA Fab fragments, resulted in 64% inhibition of attachment. These results indicated that GapA plays a role in cytadherence of M. gallisepticum to host cells.

Keywords: Mycoplasma gallisepticum, gapA gene

INTRODUCTION

Pathogenic mycoplasmas are typically non-invasive organisms and as such colonize epithelial surfaces of host tissues. Attachment to target cells is mediated by specific interactions between mycoplasma cytadhesins and their corresponding host-cell receptors (Geary & Gabridge, 1987; Geary et al., 1989). The ability of mycoplasmas to firmly adhere to cells is a prerequisite for the initiation of the processes resulting in host cell alterations and pathogenesis. Cytadherence of the human respiratory pathogen, Mycoplasma pneumoniae, involves the coordinate action of two primary cytadhesins, designated P1 (Baseman et al., 1982; Feldner et al., 1982; Hu et al., 1982, 1987) and 32 kDa protein (Baseman et al., 1987; Dallo et al., 1989b; Geary et al., 1988, 1989), and a number of accessory proteins (Krause et al., 1982, 1983) which are believed to function to regulate expression and/or localization of the cytadhesin molecules. Mycoplasma gallisepticum, Mycoplasma genitalium and Mycoplasma pirum are all located in the M. pneumoniae phylogenetic group, with M. pirum being more closely related to M. gallisepticum. It is therefore reasonable to examine these closely related species for the presence of homologous genes encoding proteins with similar functions. M. genitalium, a human mycoplasma implicated in non-gonococcal urethritis and other urogenital diseases (Maniloff et al., 1992), has been found to possess a P1 counterpart cytadhesin (Clyde & Hu, 1986; D allo et al., 1989a). This molecule, designated MgPa, possesses 48% sequence homology at the nucleotide level to P1. Shared P1 epitopes have also been identified in the 126 kDa P1-like protein of M. pirum (Tham et al., 1994).

The molecular basis of cytadherence of M. gallisepticum remains relatively unresolved. Although cytadherence function has been ascribed to numerous molecules, mainly due to biochemical and physical characterization, the genes encoding these proteins have not been analysed in detail. Previously, we described the identification of a gene from M. gallisepticum with homology to both M. pneumoniae P1 and M. genitalium MgPa genes (Goh et al., 1994; Gorton et al., 1995). These data suggest that a conserved domain (or domains) exists that is common to these three genes,

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Abbreviation: RT-PCR, reverse transcriptase-PCR.
which may be responsible for effecting the cytadherence function of each. The aim of this study was to characterize the \textit{gapA} gene and its encoded protein, and to determine the functional role of GapA in cytadherence.

**METHODS**

**Organisms and culture conditions.** \textit{Mycoplasma gallisepticum} strains S6 (gifts from Bionique Laboratories, Saranac Lake, NY, USA and Dr J. Dohms, University of Delaware, Newark, USA), PG31 (ATCC), A5969, MT-M, F (a gift from Dr. M. Tourtellotte, University of Connecticut, Storrs, USA), AAHG, R, 113-P10, 1-4P, 3T-7P, 7S-5P, 18-3P, 10-4P, 15T-3P and 29-3P (gifts from Dr S. Kleven, University of Georgia, Athens, USA) were grown in Frey’s medium (Frey et al., 1968). \textit{Mycoplasma pneumoniae} P1428 (gift from Dr M. Garbridge, Bionique Laboratories) was grown in Fortified Commercial Medium (Macy, 1980). All cultures were grown at 35 °C and harvested at 10000 g followed by three washes with sterile PBS. \textit{Mycoplasma synoviae} WVU 1853, provided by USDA-APHIS (Ames, IA, USA), was cultured in Frey’s medium supplemented with NAD. \textit{M. gallisepticum} S6 cultures were metabolically labelled by growth in Frey’s medium containing 5 μCi ml⁻¹ [³⁵S]cysteine (specific activity 1075 Ci mmol⁻¹, 398 TBq mmol⁻¹). Late-exponential-phase cultures were harvested, washed and prepared for SDS-PAGE. Separated proteins were transferred onto nitrocellulose for immunoblot analysis and autoradiography.

**PCR amplification and cloning.** Oligonucleotides flanking the P1 gene of \textit{M. pneumoniae} S6 (CAAAAACATGGCCCTGTCCA 3’ (forward) and GGGGGTTAGGGTGAGGGCGT (reverse)) were synthesized by the University of Connecticut Biotechnology Center, based on published nucleotide sequence (Su et al., 1987). PCR was performed in a total volume of 100 μl containing 1 × PCR buffer II (1 mM Tris/HCl, pH 8.3, 5 mM KCl), 250 μM each of dATP, dCTP, dGTP and dTTP, 1.5 mM MgCl₂, 350 ng of each oligonucleotide primer and 2.5 units of AmpliTaq (Applied Biosystems). The amplification conditions were as follows: 94 °C for 1 min, 60 °C for 30 s and 72 °C for 2 min for 20 cycles, with a final extension time of 10 min at 72 °C at the end of the last cycle.

The amplified \textit{M. pneumoniae} P1 gene was cloned into the pCRII TA cloning vector (Invitrogen) and transformed into \textit{E. coli} INVαF⁻ (Invitrogen) according to the manufacturer’s protocols. Plasmid DNA was isolated using the alkaline lysis procedure (Birnboim & Doly, 1979) and sequenced as described below to verify the identity of the DNA insert.

\textit{M. gallisepticum} S6 genomic DNA was digested with EcoRI, and the fragments separated by electrophoresis through a 0.8% agarose gel and treated to nitrocellulose (as described below). The blot was hybridized as described below to the P1 gene labelled with [³²P]dATP by nick translation. The 2.8 kb EcoRI fragment of \textit{M. gallisepticum} S6 which hybridized to the \textit{M. pneumoniae} P1 gene was excised and extracted from the agarose gel using the GeneClean II kit (Bio 101). It was then cloned into pBluescript SKII⁺ (Stratagene), which had been previously digested with EcoRI and treated with calf intestinal alkaline phosphatase (Gibco BRL), at a genomic DNA:vector ratio of 2:1. Ligations were performed overnight at 15 °C. Ligation mixtures were then transformed into \textit{E. coli} XL1-Blue competent cells (Stratagene) (Sambrook et al., 1989). Transformants were selected based on blue/white screening on LB plates containing 100 μg ampicillin ml⁻¹ and 25 μg X-Gal ml⁻¹. White colonies containing inserts were picked and grown in LB broth with 100 μg ampicillin ml⁻¹. Plasmid DNA from these transformants was isolated using the boiled mini-prep method (Hohnes & Quigley, 1981), followed by digestion with EcoRI, separation through a 0.8% agarose gel. DNA fragments were then transferred to BA-S85 supported nitrocellulose (Schleicher & Schuell) (Southern, 1975). The \textit{gapA} DNA was labelled with [³²P]dATP by nick translation (Sambrook et al., 1989) and hybridized to \textit{M. pneumoniae} genomic DNA at moderate stringency (37 °C with 45%, v/v, formamide) and to \textit{M. gallisepticum} genomic DNA at high stringency (42 °C with 45%, v/v, formamide) for 16 h. Blots were washed twice with 0.1% SSC, 0.1% SDS for 3 min at room temperature. The high-stringency washes at 42 °C were as for moderate stringency except for two additional washes at 50 °C in 0.16 × SSC, 0.1% SDS for 15 min. The filters were dried and autoradiographed (Fuji RX film) using intensifying screens.

**DNA sequencing.** Plasmid DNA was prepared by using the Qiagen Plasmid Maxi kit. Sequencing was performed by using the Autoread Sequencing kit (Pharmacia). The first sequencing reactions were done using the universal and reverse oligonucleotide primers specific for regions 5’ and 3’ to the cloning site on the pBluescript SKII⁺ and pCRII vectors. The remaining sequence was determined by either primer walking utilizing fluorescein-labelled oligonucleotide primers complementary to newly determined sequence or using universal and reverse primers on nested deletion clones generated utilizing the Erase-a-Base system (Promega). Both DNA strands have been sequenced independently. Nucleotide and deduced amino acid sequence analyses were carried out using the MacDNASIS, BESTFIT and BLAST (Altschul et al., 1990) programs.

**Generation of antiserum.** A 14 amino acid peptide (LQAN-KKDGAASSPSK) was selected from the \textit{gapA} deduced amino acid sequence, synthesized and coupled to keyhole limpet haemocyanin (Bio-synthesis). Rabbit preimmune serum was collected, then the rabbit was immunized subcutaneously with 1 mg synthetic GapA peptide in Freund’s complete adjuvant. The rabbit was boosted four times at 3-week intervals with 177 nmol GapA peptide in Freund’s incomplete adjuvant. Blood was collected from the ear vein 7 d after the last immunization. Anti-peptide serum was used to identify clones containing inserts that hybridized with the P1 gene. The \textit{M. gallisepticum} fragments that hybridized with P1 were referred to as \textit{gapA}.

**Southern blot analysis.** Mycoplasma genomic DNAs were extracted (Hempstead, 1990) and digested with various restriction enzymes according to the manufacturer’s recommendations (Gibco BRL), and 5 μg of each was electro-phoresed through a 0.8% agarose gel. DNA fragments were then transferred to BA-S85 supported nitrocellulose (Schleicher & Schuell) (Southern, 1975). The \textit{gapA} DNA was labelled with [³²P]dATP by nick translation (Sambrook et al., 1989) and hybridized to \textit{M. pneumoniae} genomic DNA at moderate stringency (37 °C with 45%, v/v, formamide) and to \textit{M. gallisepticum} genomic DNA at high stringency (42 °C with 45%, v/v, formamide) for 16 h. Blots were washed twice with 0.1% SSC, 0.1% SDS for 3 min at room temperature. The high-stringency washes at 42 °C were as for moderate stringency except for two additional washes at 50 °C in 0.16 × SSC, 0.1% SDS for 15 min. The filters were dried and autoradiographed (Fuji RX film) using intensifying screens.
Zealand White rabbits. The rabbits were immunized according to the same procedure as that used to produce the anti-peptide serum.

**SDS-PAGE and immunoblot analysis.** *M. gallisepticum* proteins were separated in 7% SDS-polyacrylamide gels under reducing conditions (Laemmli, 1970), transferred onto nitrocellulose (MSI) (Towbin et al., 1979) and stained with Ponceau S (Sigma) to visualize the transferred proteins. The membranes were blocked with 5% BSA, 20% foetal bovine serum in Tris-buffered saline (10 mM Tris, pH 7.4, 0.9% NaCl) for 1 h at 37°C, then reacted with either rabbit anti-peptide serum (1:250) or rabbit anti-GapA(1:8000) serum for 1 h at 37°C with gentle rocking. After washing three times with PBS containing 0.5% Tween 20, horseradish-peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories) (1:7000) was added and the membranes incubated for 1 h at 37°C with rocking. The membrane was washed three times as before and then developed with 4-chloro-1-naphthol and hydrogen peroxide.

**Colony immunoblotting.** Nitrocellulose membranes (82 mm diameter, 0.45 μM pore size) (MSI) were placed onto *M. gallisepticum* colonies grown on agar plates. The membranes were removed after 5 min and blocked with 3% BSA in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, (TS buffer) for 1 h at room temperature with gentle rocking (Rosengarten & Yoge, 1996). These were washed three times in TS buffer at 5 min intervals. Rabbit anti-GapA serum (1:8000) was added and incubated overnight at 4°C. The membranes were washed three times as before, then horseradish peroxidase conjugated goat anti-rabbit IgG (1:7000) was added and the blots incubated at room temperature for 1 h. The reactions were visualized using 4-chloro-1-naphthol and hydrogen peroxide.

**Trypsin-sensitivity assay.** Trypsinization assays were performed according to a modification of the methods of Layh-Schmitt & Herrmann (1992) and Franzoso et al. (1993). Cells from a exponential-phase culture of *M. gallisepticum* S6 were pelleted and washed as described above. The cells were resuspended to a protein concentration of 2.5 mg ml⁻¹. Trypsin (Difco) was added to 2 ml cells to a final concentration of 200 μg ml⁻¹, and the cells incubated at 37°C. Samples (300 μl) were removed after 5, 10, 20, 30 and 60 min incubation and boiled for 5 min to inactivate the trypsin. Untreated *M. gallisepticum* S6 cells were used as a negative control. All samples were then subjected to SDS-PAGE and anti-GapA immunoblot analysis as described above.

**Reverse transcriptase-PCR (RT-PCR).** *M. gallisepticum* RNA was isolated by the hot phenol extraction method (Sambrook et al., 1989). The GeneAmp RNA PCR kit (Applied Biosystems) was used for cDNA synthesis. Reverse transcription was done in a total volume of 20 μl containing 5 mM MgCl₂, 1 mM each dNTP, 1 unit RNase inhibitor, 2.5 units MuLV reverse transcriptase, 2.5 μM random hexamers and 1 μg *M. gallisepticum* RNA. The reactions were incubated for 10 min at room temperature for extension of the hexamers on target RNA, followed by 15 min at 42°C, denaturation at 99°C for 5 min and cooling at 5°C for 5 min. MuLV reverse transcriptase was omitted from the negative control samples to ensure that no genomic DNA was present in the target samples. PCR amplification was performed on these samples in a total volume of 100 μl containing 2 mM MgCl₂, 2.5 units AmpliTaq and 350 ng each oligonucleotide using 35 cycles of 94°C for 1 min, annealing at 5°C below the melting temperature of the oligonucleotides used (see Fig. 4) for 1 min and 72°C for 2 min, followed by a final extension at 72°C for 10 min.

**Generation of Fab fragments.** Immunoglobulins were precipitated from serum samples using ammonium sulfate as described by Forsyth et al. (1992). Fab fragments of anti-GapA were generated by digestion of 5 mg antibody ml⁻¹ with papain conjugated to agarose beads (25 units ml⁻¹) at 37°C for 18 h. The digestion was terminated by removal of the enzyme by centrifugation at 5000 g for 5 min. The Fab fragments were separated from the Fc fragments using *Staphylococcus aureus* Protein A.

**M. gallisepticum inhibition-of-attachment assay.** This assay was performed according to the procedure described by Forsyth et al. (1992). Chicken tracheal rings, obtained from 9-week-old specific-pathogen-free White Leghorn chickens, were cut into 1 cm sections. The rings were selected at random (to minimize potential attachment-site preferences in the trachea) and one end embedded in agarose in 24-well tissue culture plates. *M. gallisepticum* S6, labelled with L-[¹⁴C]amino acids (specific activity, 534 mCi mmol⁻¹, 1.98 GBq mmol⁻¹) at 2 μCi ml⁻¹, were harvested by centrifugation at 10000 g followed by three washes with serum-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) and resuspended in one-tenth of the original culture volume. Resuspended *M. gallisepticum* (50 μl) was incubated with 50 μl (30 μg) of either Fab fragments derived from preimmune serum (negative control) or anti-GapA Fab fragments at 37°C for 1 h. Fifty microlitres of each mixture was placed into individual tracheal ring sections. After 1 h incubation at 37°C, the tracheal rings were washed with serum-free DMEM, placed into individual scintillation vials containing 5 ml scintillation fluid and the radioactivity measured in a scintillation counter. Seven tracheal rings were used per sample and the experiment was repeated three times.

**RESULTS**

**Southern blot analysis**

A 4.7 kb fragment of the *M. pneumoniae* P1 gene was amplified by PCR. After its identity was confirmed by DNA sequencing, restriction enzyme analysis and Southern blot hybridization, the P1 gene was labelled with [³²P]dATP and used as a probe. Hybridization of *M. gallisepticum* S6 DNA (5 μg) digested with BanHI, EcoRI, HindIII or PstI with the P1 gene under moderate stringency indicated the presence of a gene with homology to P1. The P1 probe hybridized to single 6.0 kb HindIII, 2.8 kb EcoRI and 3.5 kb PstI fragments in *M. gallisepticum* S6, indicating that the gapA gene existed as a single copy in the genome.

The 2.8 kb EcoRI fragment of *M. gallisepticum*, cloned into pBluescript and labelled with [³²P]dATP, was used to probe other strains of *M. gallisepticum* by Southern hybridization at high stringency. Analysis of *M. gallisepticum* genomic DNA digested with EcoRI, PstI, HindIII or BanHI showed that the probe hybridized to fragments of similar size to those predicted from the restriction enzyme map of the nucleotide sequence and showed that the gapA gene was present in all of the *M. gallisepticum* strains examined including S6, F, R, A969, AAHG, PG31, 113P-10 (Fig. 1a), 1-4P, 3T-7P, 6-3P, 7S-5P, 18-3P, 10-4P, 15T-3P, 29-3P and MTM (not shown). Variations in the observed sizes of the HindIII fragments hybridizing to gapA are probably due to
sequence variations outside of the gapA ORF. Three fragments (approximately 6.0, 5.3 and 0.7 kb) hybridized to the gapA probe in strain PG31 but in no other strain examined. Fifty single colonies of PG31 were randomly selected, grown and subjected to DNA extraction. PCR amplifications followed by HindIII digestion of these 50 clones showed that six (12%) had an internal HindIII site in the gapA gene. Hybridization demonstrated that a subpopulation contained gapA on two fragments (5.3 and 0.7 kb), while the majority of the population contained the entire gapA gene within a single 6.0 kb fragment (Fig. 1b). The gapA gene was cloned and sequenced from these two subpopulations. The internal HindIII site was found to be due to a base change from GAT to TAT, resulting in an amino acid substitution of aspartic acid to tyrosine. This base change did not result in a frame shift or affect the expression of the GapA protein.

The gapA gene hybridized with only the 5.6 kb EcoRI fragment of M. pneumoniae DNA, which contains the complete P1 gene, but did not hybridize with any of the repeat elements found in the P1 gene. The gapA gene did not hybridize to M. synoviae WWU1853 DNA even under low stringency, and was not able to be amplified from M. synoviae genomic DNA by PCR, using gapA-specific primers, indicating that a homologous gene was not present in M. synoviae.

Sequence and RT-PCR analysis

The gapA gene is a 2895 bp ORF, encoding a predicted 105 kDa protein. Analysis of the gapA nucleotide sequence revealed it possesses 64 mol% A + T and has an overall identity of 45% to the P1 gene, 46% to the MgPa gene and 47% to the P1-like protein gene of M. pirum. The deduced amino acid sequence of GapA was analysed and compared to the P1 and the MgPa amino acid sequences using the BESTFIT program. This analysis indicated that GapA had significant overall amino acid homology with P1 (50% similarity, 29% identity) and MgPa (48% similarity, 27% identity). The greatest degree of amino acid homology was in a 138 amino acid stretch of the carboxyl terminus. This region possessed 59% similarity and 33% identity to the P1 gene. It was found to be proline rich and 13 of 14 tryptophan codons were TGA. Autoradiography of [35S]cysteine-labelled M. gallisepticum S6 proteins following Triton X-114 phase partitioning and SDS-PAGE separation (Fig. 2) verified that GapA contained cysteine.

Based on comparison with published putative P1-binding sites (Gerstenecker & Jacobs, 1990; Jacobs et al., 1989; Dallo et al., 1988), homologous domains were located in GapA. Three possible domains were identified at positions 182–213, 1102–1144 and 2310–2330 of the gapA gene. Region 2310–2330 showed 53% homology to the binding domain described in the middle of the P1
Mycoplasma gallisepticum cytadhesin GapA

Fig. 3. Hydropathy profiles of (a) M. pneumoniae P1, (b) M. genitalium MgPa and (c) M. gallisepticum GapA carboxyl termini as determined by the method of Kyte & Doolittle (1982). Positive values on the y axis indicate hydrophobic regions and negative values indicate hydrophilic regions of the protein. The numbers on the x axis indicate the amino acids.

Comparison of the hydropathy profile of the 138-amino-acid carboxyl terminus of GapA to that of the carboxyl termini of P1 and MgPa revealed striking similarities (Fig. 3). All three possessed an initial hydrophobic region, predicted to be membrane associated, followed by short alternating hydrophilic and hydrophobic regions. All three terminated with proline-rich regions, which in the case of P1 has been shown to be facing the cytoplasmic compartment (Razin & Jacobs, 1992).

Examination of the sequences immediately flanking the gapA gene suggested that the gapA ORF was located within an operon. Six primer sets designed to amplify overlapping regions of the 6·0 kb HindIII fragment containing gapA were used in RT-PCR analysis (Fig. 4). Primer set 1 amplified a 1275 bp product 5' to gapA. Primer sets 2 and 3 had one primer of each set anchored within gapA and the other 5' to gapA. These resulted in 1020 and 1527 bp products, respectively, which overlapped with each other and with the amplicon from primer set 1. Primer set 4 amplified a 1334 bp product totally within gapA. Primer sets 5 and 6 had one primer of each pair anchored within gapA and the other corresponding primer 3' to gapA. These resulted in 1059 and 812 bp overlapping amplicons. A potential ribosome-binding site was found between bases −32 and −25 upstream of the gapA start site. No consensus promoter was found immediately upstream of the gapA start codon, no termination sequences are present in the immediately adjacent regions and overlapping products obtained from RT-PCR amplifications indicated that gapA was transcribed as part of a larger polycistronic message.

A 3 kb HindIII–PstI fragment 5' of gapA was cloned and sequenced. This sequence had 45% homology with ORF4 of the P1 operon, which encodes a 28 kDa protein. A potential third ORF (ORF3) was found downstream of gapA, as suggested by a start codon 24 bp from the gapA stop codon. Partial DNA sequence analysis of this ORF revealed no sequence homology with the P1 operon.

Protein characterization

Anti-GapA immunoblot analysis of M. gallisepticum resulted in the recognition of a 105 kDa protein in strains S6 and A5969, a 110 kDa protein in strains R and PG31, and an approximately 98 kDa protein in strain F (Fig. 5). Colony immunoblots resulted in positive reactions, indicating that GapA was surface exposed (data not shown). Immunoblots of M. synoviae using anti-GapA serum were negative.

Immunoblot analysis of trypsin-treated M. gallisepticum using anti-GapA serum showed that GapA was degraded after 5 min digestion and was no longer detected after 20 min. These results indicated that GapA was a surface-exposed, trypsin-sensitive protein.

Incubation of 14C-labelled M. gallisepticum S6 with 20 µg rabbit anti-GapA Fab fragments resulted in a mean 64% inhibition (P < 0·01) of attachment to chicken tracheal-ring epithelial cells, compared to incubation with 20 µg Fab fragments derived from the preimmune serum.

DISCUSSION

The initial and most crucial event in the establishment of disease by M. gallisepticum is the successful interaction between the organisms and the host cell. To understand the mechanisms involved, it is important to identify the cytadhesins and related accessory factors participating in this complex process. Conservation of antigenic determinants have been described among M. pneumoniae, M. genitalium and M. gallisepticum (Clyde & Hu, 1986). It has been shown that the 170 kDa P1 cytadhesin of M. pneumoniae shares common epitopes with the 140 kDa MgPa cytadhesin of M. genitalium.
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**Fig. 4.** Physical map of the gapA operon. (a) RT-PCR analysis of the gapA operon. (b) Linear restriction map of the gapA operon. B, BamHI; E, EcoRI; H, HindIII; P, PstI. (c) Primer sequences used for RT-PCR analysis of gapA operon and the resulting amplicon sizes.

**Fig. 5.** Immunoblot analysis of *M. gallisepticum* strains using anti-GapA serum. Lanes: 1, A5969; 2, AAHG; 3, F; 4, R; 5, PG31; 6, S6. The arrow indicates GapA.

(Morrison-Plummer *et al.*, 1987) and the 126 kDa P1-like protein of *M. pirum* (Tham *et al.*, 1994). Although this P1-like protein shares conserved epitopes with the above-mentioned cytadhesins, it has not been shown to function as a cytadhesin. In this study, we have determined that the 105 kDa GapA of *M. gallisepticum* shares epitopes conserved within both of the confirmed cytadhesins, P1 and MgPa, and itself functions as a cytadhesin.

The gapA ORF is 2895 bp and is predicted to encode a protein with a molecular mass of 105 kDa. It has a 64 mol% A+T content, compared to 46 mol% for P1, 60 mol% for MgPa (Dallo *et al.*, 1989b) and 72 mol% for the P1-like protein of *M. pirum* (Tham *et al.*, 1994). In this regard it is more similar to the P1 homologues, MgPa and the P1-like protein, than P1 itself. However, gapA and its product exhibit numerous characteristics similar to those of P1. Nucleotide sequence analysis of the gapA ORF revealed 45% identity to the P1 gene and 46% identity to the MgPa gene. Similar to P1 and MgPa (Dallo *et al.*, 1989a; Hu *et al.*, 1987; Inamine *et al.*, 1988a, 1989), GapA has a high proline content, located predominantly at the carboxyl terminus. This proline-rich region has been suggested to affect the conformation of the polypeptide chain in such a manner as to aid in the topological organization of the cytadhesin (Razin & Jacobs, 1992). Both the P1 and MgPa genes are located within three-gene operons (Inamine *et al.*, 1988a, b, 1989); the gapA gene also appears to be located within an operon.

Two cysteine residues are found in the amino-terminal region of GapA, based on the deduced amino acid sequence. Keeler *et al.* (1996) reported that a 150 kDa protein (MGC1) is a P1-like protein in *M. gallisepticum*, although the largest possible ORF found in the nucleotide sequence would encode a predicted 120 kDa protein. *M. gallisepticum* S6 was obtained from Dr. J. Dohms and confirmed by RAPD analysis (Geary *et al.*, 1994), RFLP analysis and protein profiles to be *M. gallisepticum* S6. Their S6 and the S6 used in our laboratory were analysed by immunoblotting using both anti-GapA serum and antiserum to a GST fusion protein of MGC1 (a gift from Dr. J. Dohms). The results showed that both antisera recognized a 105 kDa protein in both S6 cultures, and not a 150 kDa protein. Keeler *et al.* (1996) also stated that MGC1 was devoid of cysteine. The nucleotide sequences of gapA and mgc1 differ only in the 5' region of the gene where the predicted cysteine

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**Table 1.** Fragment Size (bp) Primer set/position Forward primers Reverse primers

<table>
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<th>Fragment</th>
<th>Size (bp)</th>
<th>Primer set/position</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tr>
<td>a</td>
<td>1275</td>
<td>1 (1022 – 2297)</td>
<td>5'AAACACCTAACCCACCTAC3'</td>
<td>5'AGGAATGAATCAACCCCC3'</td>
</tr>
<tr>
<td>b</td>
<td>1020</td>
<td>2 (2121 – 3141)</td>
<td>5'AGACCAAACCTCCTAACC3'</td>
<td>5'AGGAATGAATCAACCCCC3'</td>
</tr>
<tr>
<td>c</td>
<td>1527</td>
<td>3 (2776 – 4303)</td>
<td>5'GCCGGATTGATTTGTATG3'</td>
<td>5'GGAAACACAAAACAAGT3'</td>
</tr>
<tr>
<td>d</td>
<td>1334</td>
<td>4 (4269 – 5603)</td>
<td>5'AllAGTAAGCCAGCTGGT3'</td>
<td>5'CAATGTCTCAAAACCGTAAG3'</td>
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<tr>
<td>e</td>
<td>812</td>
<td>6 (5951 – 6764)</td>
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<td>5'ACCTGGGCAAACAAAAT3'</td>
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<td>f</td>
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<td>5 (51 74 – 6233)</td>
<td>57AATGTAATCGGTCAAGGTGC3'</td>
<td>5'CTAAGTGATGA~GCTGG3'</td>
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is found. To assess for the presence of cysteine in the GapA protein, [35S]cysteine was used to metabolically label M. gallisepticum S6 followed by protein separation by SDS-PAGE and autoradiography. The results indicated that GapA contained cysteine.

The gapA gene was found to exist in many different strains of M. gallisepticum. PCR amplification, Southern hybridization and immunoblot analysis all indicated that a P1 homologue was not present in M. synoviae. RFLP and Southern blot analysis indicated that gapA existed as a single copy in all M. gallisepticum strains assessed, and RT-PCR analysis indicated that it was transcribed in all as well. This single-copy nature contrasts with the M. pneumoniae P1 and M. genitalium MgPa genes, which are present in multiple copies throughout the genome (Su et al., 1988; Dallo & Baseman, 1991).

The results of the immunoblot analysis on the various strains indicated a specific reaction with only GapA and demonstrated an intraspecies strain variation in the size of GapA, with observed molecular masses of approximately 98, 105 and 110 kDa in different strains. Internal amino acid analysis of the 110 kDa form of GapA in strain R revealed 100% identity to the amino acid sequence of the 105 kDa product in S6. Sequence analysis from other strains is under way so that we may understand the genetic basis for this strain variation.

To assess the role of GapA in the cytadherence process of M. gallisepticum, the chicken tracheal-ring inhibition-of-attachment assay was used. Compared to the control preimmune Fab fragments, anti-GapA Fab fragments were shown to be capable of inhibiting M. gallisepticum attachment by 64%, indicating a distinct role in adherence to host cells. Further studies are necessary to determine the functional interaction of GapA with the other major cytadhesin of M. gallisepticum, LP64 (Forsyth et al., 1992).

The proteins encoded by genes flanking the P1 gene have been suggested to have cytadherence-related functions. It remains to be determined whether the ORF upstream of gapA, which has 45% homology with ORF4 of the P1 operon, encodes a homologue of the 28 kDa M. pneumoniae protein or has any cytadherence-related function.

Investigations into the nature of homologous genes from related species of mycoplasmas may provide insights into the conservation and/or modifications of genes essential for survival or pathogenesis throughout the process of degenerate evolution which is characteristic for mycoplasmas. As our understanding of the complex mechanism of cytadherence increases, a more efficacious vaccine may also be designed for the control of M. gallisepticum disease, utilizing the cytadherence molecules as subunit antigens.

ACKNOWLEDGEMENTS

This research was supported in part by USDA grant 94-37208-1069 (S. J. G), USDA Agricultural Experiment Station grant CONSOO640 (S. J. G) and Sigma Xi grant 10102 (M. S. G).

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Received 1 May 1998; accepted 9 July 1998.