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

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The identification of a gene (*gapA*) from *Mycoplasma gallisepticum* with homology to the P1 cytoadherence 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 cytoadherence 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 cytoadhesins 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. Cytoadherence of the human respiratory pathogen, *Mycoplasma pneumoniae*, involves the coordinate action of two primary cytoadhesins, 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 cy-

adhesin 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 cytoadhesin (Clyde & Hu, 1986; Dallo *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 cytoadherence of *M. gallisepticum* remains relatively unresolved. Although cytoadherence 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.

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which may be responsible for effecting the cytoadherence function of each. The aim of this study was to characterize the *gapA* gene and its encoded protein, and to determine the functional role of GapA in cytoadherence.

METHODS

Organisms and culture conditions. *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, 6-3P, 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). *Mycoplasma pneumoniae* P11428 (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. *Mycoplasma synoviae* WVU 1853, provided by USDA-APHIS (Ames, IA, USA), was cultured in Frey's medium supplemented with NAD. *M. gallisepticum* S6 cultures were metabolically labelled by growth in Frey's medium containing 5 µCi ml⁻¹ L-[³⁵S]cysteine (specific activity 1075 Ci mmol⁻¹, 39.8 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 *M. pneumoniae* [5' CAAAAAACTGCCCTGTCCA 3' (forward) and 5' GGGCGTGTAGGGGGTGAGGG 3' (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 *M. pneumoniae* P1 gene was cloned into the pCRII TA cloning vector (Invitrogen) and transformed into *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.

M. gallisepticum S6 genomic DNA was digested with *Eco*RI, and the fragments separated by electrophoresis through a 0.8% agarose gel and transferred to nitrocellulose (as described below). This blot was hybridized as described below to the P1 gene labelled with [³²P]dATP by nick translation. The 2.8 kb *Eco*RI fragment of *M. gallisepticum* S6 which hybridized to the *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 *Eco*RI 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 *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 (Holmes & Quigley, 1981), followed by digestion with *Eco*RI, separation through a 0.8% agarose gel, Southern transfer and probing with the *M. pneumoniae* P1 gene to identify clones containing inserts that hybridized with the P1 gene. The *M. gallisepticum* fragments that hybridized with P1 were referred to as *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 electrophoresed through a 0.8% agarose gel. DNA fragments were then transferred to BA-S85 supported nitrocellulose (Schleicher & Schuell) (Southern, 1975). The *gapA* DNA was labelled with [³²P]dATP by nick translation (Sambrook *et al.*, 1989) and hybridized to *M. pneumoniae* genomic DNA at moderate stringency (37 °C with 45%, v/v, formamide) and to *M. gallisepticum* genomic DNA at high stringency (42 °C with 45%, v/v, formamide) for 16 h. Blots were washed twice with 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), 0.1% SDS and 0.2 × 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-KKDGASSPSK) was selected from the *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 500 µg synthetic GapA peptide in Freund's incomplete adjuvant. Blood was collected from the ear vein 7 d after the last immunization. Anti-peptide serum was then used to identify the *M. gallisepticum* protein encoded by the *gapA* gene, as described below. The 105 kDa protein band that reacted with anti-peptide serum was excised and electroeluted from a polyacrylamide gel using the Bio-Rad Electroelution unit. The eluted protein was run into a 10% SDS-PAGE gel and a portion immunostained (as described below) with the anti-peptide serum. The protein was also subjected to in-gel digestion with Lys-C protease followed by sequence analysis (Edman & Begg, 1967). The internal amino acid sequence data in conjunction with the immunoblot data were used to verify the purity and identity of the protein prior to immunizing New

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 & Yogeve, 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 an 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, 53.4 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 *Bam*HI, *Eco*RI, *Hind*III or *Pst*I 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 *Hind*III, 2.8 kb *Eco*RI and 3.5 kb *Pst*I fragments in *M. gallisepticum* S6, indicating that the *gapA* gene existed as a single copy in the genome.

The 2.8 kb *Eco*RI 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 *Eco*RI, *Pst*I, *Hind*III or *Bam*HI 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, A5969, 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 *Hind*III fragments hybridizing to *gapA* are probably due to

Fig. 1. Hybridization of *Hind*III-digested DNA from various strains of *M. gallisepticum* with 32 P-labelled *M. gallisepticum* *gapA*. (a) Lane 1, S6; lane 2, A5969; lane 3, F; lane 4, R; lane 5, PG31; lane 6, AAHG; lane 7, 113P-10. (b) Lane 1, PG31 mixed population; lane 2, PG31 clone 2; lane 3, PG31 clone 8.

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 *Hind*III digestion of these 50 clones showed that six (12%) had an internal *Hind*III 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 *Hind*III 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 *Eco*RI 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* WVU1853 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

Fig. 2. Right: autoradiography of Triton X-114 partitioned 35 S-cysteine-labelled *M. gallisepticum* S6 proteins. Lanes: 1, insoluble phase; 2, aqueous phase; 3, detergent phase. Left: immunoblot analysis of the 35 S-cysteine-labelled *M. gallisepticum* S6 insoluble phase was performed using: A, preimmune rabbit serum and B, anti-GapA serum. The arrow indicates the 35 S-cysteine-labelled GapA in the insoluble fraction and the corresponding immunoblot.

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 35 S-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

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.

gene (Jacobs *et al.*, 1989). Region 182–213 showed 47% homology to the putative amino terminal binding domain and 1102–1144 showed 56% homology to the 13-amino-acid binding domain sequence described by Dallo *et al.* (1988).

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 ^{14}C -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 cytoadhesins 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 cytoadhesin of *M. pneumoniae* shares common epitopes with the 140 kDa MgPa cytoadhesin of *M. genitalium*

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, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I. (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 cytoadhesins, it has not been shown to function as a cytoadhesin. In this study, we have determined that the 105 kDa GapA of *M. gallisepticum* shares epitopes conserved within both of the confirmed cytoadhesins, P1 and MgPa, and itself functions as a cytoadhesin.

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 cytoadhesin (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

is found. To assess for the presence of cysteine in the GapA protein, [^{35}S]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 cytoadherence 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 cytoadhesin of *M. gallisepticum*, LP64 (Forsyth *et al.*, 1992).

The proteins encoded by genes flanking the P1 gene have been suggested to have cytoadherence-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 cytoadherence-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 cytoadherence increases, a more efficacious vaccine may also be designed for the control of *M. gallisepticum* disease, utilizing the cytoadherence molecules as subunit antigens.

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