A gene family in *Mycoplasma imitans* closely related to the pMGA family of *Mycoplasma gallisepticum*

Philip F. Markham, Michael F. Duffy,[†] Michelle D. Glew[‡] and Glenn F. Browning

Author for correspondence: Philip F. Markham. Tel: +61 3 9344 7368. Fax: +61 3 9344 7374.
e-mail: p.markham@vet.unimelb.edu.au

Department of Veterinary Science, The University of Melbourne, Parkville, Victoria, Australia 3052

The avian pathogen *Mycoplasma gallisepticum* possesses a large gene family encoding lipoproteins which function as haemagglutinins. Representative species of the pneumoniae phylogenetic group of mycoplasmas were examined for the presence of genes homologous to members of this multigene family. Antisera against the pMGA1.1 lipoprotein recognized a 35 kDa protein in *Mycoplasma imitans*, but did not recognize proteins of *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Mycoplasma pirum*, *Mycoplasma penetrans* or *Mycoplasma iowae* in Western blots. A fragment of the pMGA1.2 gene and oligonucleotide probes complementary to highly conserved coding and non-coding regions of pMGA genes bound to fragments of genomic DNA of *M. imitans*, but not to the genomes of *M. genitalium*, *M. pneumoniae*, *M. pirum* or *M. penetrans*, and only one probe bound to a fragment of the *M. iowae* genome. One homologue of the pMGA genes was amplified from the *M. imitans* genome by PCR and used as a probe to clone a 3.1 kbp DNA fragment from a library of *Hind*III-digested *M. imitans* genomic DNA. The contiguous DNA sequence of the PCR and *Hind*III clones was predicted to encode one complete and one partial ORF which shared some peptide sequence identity with the pMGA genes, including the signal peptidase II cleavage site and the proline-rich amino-terminal region. Like the pMGA genes, the *M. imitans* genes were found to be members of a large gene family, with an association with GAA trinucleotide repeats, a feature which distinguishes these two families from the homologous *vlhA* gene family in *Mycoplasma synoviae*. The identification of these gene families in three phylogenetically distinct avian mycoplasma species, but not in human mycoplasmas, suggests their horizontal transfer between species infecting the same host.

Keywords: pMGA homologue, *Mycoplasma imitans*, multigene family

INTRODUCTION

Mycoplasma gallisepticum possesses a large multigene family, with family members encoding a cell-surface-exposed lipoprotein (pMGA) involved in haemag-

glutination (Markham *et al.*, 1992, 1994). Analysis of several *M. gallisepticum* strains revealed that each possessed a repertoire of between 32 and 70 pMGA genes (Baseggio *et al.*, 1996). Several features are highly conserved between pMGA genes, including the presence of the trinucleotide repeat GAA that precedes the transcriptional start of each gene, and the region encoding the leader sequence preceding the amino-terminal cysteine of the mature peptide. It has been suggested that the pMGA gene family in *M. gallisepticum* may be involved in immune evasion and thus aid the continued survival of the organism in the host

[†] Present address: Walter and Elisa Hall Institute for Medical Research, Parkville, Victoria, Australia 3052.

[‡] Present address: Institute of Bacteriology and Hygiene, Vienna University of Veterinary Medicine, A-1210, Vienna, Josef Baumann-Gasse 1, Austria.

The GenBank accession number for the sequence in this paper is AF141940.

(Markham *et al.*, 1993; Baseggio *et al.*, 1996). Recent studies have also identified a homologous gene family in a phylogenetically distant avian pathogen, *Mycoplasma synoviae*, although the GAA trinucleotide repeat sequence shown to control pMGA gene transcription in *M. gallisepticum* (Glew *et al.*, 1998) was not associated with the *M. synoviae* family (Noormohammadi *et al.*, 1998).

Phylogenetic studies of mycoplasmas using the 16S rRNA gene have placed *M. gallisepticum* within the pneumoniae group (Weisburg *et al.*, 1989). This group comprises a number of species including *Mycoplasma pneumoniae*, *Mycoplasma penetrans*, *Mycoplasma genitalium*, *Mycoplasma pirum*, *Mycoplasma iowae*, *Mycoplasma muris* and *Ureaplasma urealyticum* (Behbahani *et al.*, 1993). The pneumoniae group also contains another avian mycoplasma closely related to *M. gallisepticum*, both serologically and genetically (Bradbury *et al.*, 1993), and analysis of the 16S rRNA gene sequence of *Mycoplasma imitans* strain 4229 (ATCC 51306) revealed its closest known neighbour was *M. gallisepticum* (Boyle & Morrow, 1994). Recent investigations have shown that cytoadherence of *M. imitans* to avian embryo tracheal organ cultures resulted in ciliostasis (Abdul *et al.*, 1996), and that it causes respiratory disease in red-legged partridges (Ganapathy & Bradbury, 1998) and in turkey poult infected with rhinotracheitis virus (Ganapathy *et al.*, 1996).

Studies of three members of the pneumoniae group (*M. pneumoniae*, *M. genitalium*, *M. gallisepticum*), using hyperimmune sera raised against the P1 attachment protein of *M. pneumoniae* and nucleic acid probes for its gene, identified homologous proteins and genes in both *M. genitalium* and *M. gallisepticum*, suggesting the existence of P1 homologues in these species (Dallo & Baseman, 1990). The presence of P1-like genes in *M. gallisepticum* and *M. genitalium* has been confirmed with the cloning and sequencing of MGC1 (Keeler *et al.*, 1996; Goh *et al.*, 1998) and MgPa (Inamine *et al.*, 1989), respectively, and a homologue has also been identified in *M. pirum* (Tham *et al.*, 1994). Each gene encodes a protein with similar structural features to P1.

Considering the importance of pMGA in *M. gallisepticum* it would be of interest to know whether pMGA is found in other members of the pneumoniae group, and if it is, whether the trinucleotide repeat which functions as a transcriptional control motif is also present in other species. The aim of this study was to examine several members of the pneumoniae group, including *M. imitans*, *M. iowae*, *M. genitalium*, *M. pirum*, *M. penetrans* and *M. pneumoniae*, for proteins homologous to pMGA and for gene families which may encode them.

METHODS

Bacterial strains and growth conditions. The media and conditions for growth of strain S6 of *M. gallisepticum* have been previously described (Markham *et al.*, 1992). *M. imitans*

4229 and *M. iowae* serovar J were obtained from C. J. Morrow (origin described by Boyle *et al.*, 1995) and cultured as for *M. gallisepticum*. *M. genitalium* (ATCC 33530), *M. pirum* (ATCC 25960), *M. penetrans* (ATCC 55252) and *M. pneumoniae* strains M129 (ATCC 29342) and P1428 (ATCC 29085) were obtained from the American Type Culture Collection. *M. pneumoniae* strain FH was a kind gift from V. Peters of the Department of Virology, Royal Children's Hospital, Melbourne. *M. genitalium*, *M. pirum*, *M. penetrans* and *M. pneumoniae* strains were propagated in spiroplasma medium SP4 containing 17% heat-inactivated foetal bovine serum at 37 °C with 5% CO₂. *M. pirum* was cultured in ATCC medium 243 supplemented with 20% heat-inactivated horse serum at 37 °C with 5% CO₂.

Escherichia coli strain DH5 α was used as host for recombinant plasmids as described previously (Markham *et al.*, 1993).

Isolation of mycoplasma DNA. Mycoplasma cells were grown in broth medium until a decrease in pH of the media had occurred or in the case of *M. pirum*, until an increase in turbidity was observed. Mycoplasma cells were pelleted by centrifugation at 20000 g for 20 min at 4 °C and the cell pellet was resuspended in PBS (0.1 M phosphate, 0.33 M NaCl, pH 7.4). Cells were washed twice more in PBS. The method used for extraction of DNA from *M. gallisepticum*, *M. imitans* and *M. iowae* has been described previously (Kleven *et al.*, 1988). For extraction of DNA from *M. genitalium*, *M. pirum*, *M. penetrans* and *M. pneumoniae* a modification of the method of Su *et al.* (1988) was used as overnight incubations for extraction of DNA resulted in degradation of the DNA. Briefly, cell pellets of *M. genitalium*, *M. pirum*, *M. penetrans* and *M. pneumoniae* were resuspended in 0.5% SDS, 50 mM EDTA, 50 mM Tris, pH 8.0 (HCl), to which 50 µg proteinase K ml⁻¹ (Boehringer Mannheim) was added and the mixture was incubated at 50 °C for 2 h. RNase A (Boehringer Mannheim) was added to give a concentration of 50 µg ml⁻¹ and the solution was incubated for a further 30 min at 37 °C. The mixture was extracted sequentially with an equal volume of phenol (ICN Biomedicals) buffered to pH 6.7, twice with an equal volume of 50% phenol/50% 24:1 (v/v) chloroform/isoamyl alcohol and twice with an equal volume of 24:1 (v/v) chloroform/isoamyl alcohol. Following extraction, 3 M sodium acetate (pH 7.0) was added to the mixture to a final concentration of 0.25 M. The DNA was precipitated by adding 2 vols ice-cold ethanol and incubating the solution at -20 °C overnight. The DNA was removed by swirling a glass rod through the liquid. The recovered DNA was air-dried and redissolved in 10 mM Tris, pH 8.0 (HCl), 1.0 mM EDTA overnight at 4 °C.

SDS-PAGE and immunoblotting. Mycoplasma cells were grown and harvested as described above. The washed cell pellet was resuspended in 1× SDS-PAGE lysis buffer [0.125 M Tris, pH 6.8 (HCl), 4% (w/v) SDS, 20% glycerol, 0.05% bromophenol blue] and the proteins in a sample were separated by electrophoresis through a 10% polyacrylamide gel and either stained with Coomassie brilliant blue or transferred onto Immobilon (Millipore) membrane as described previously (Markham *et al.*, 1992). Immunostaining of the membrane was performed essentially as described before (Markham *et al.*, 1992). Briefly, the membrane was incubated in PBS containing 10% bovine serum albumin (10% BSA/PBS) for 2 h at room temperature with gentle rocking, then washed three times (10 min each) in 0.1% BSA/PBS containing 0.05% Tween 20. The membrane was incubated for 1 h at room temperature with polyclonal rabbit antisera raised against pMGA1.1 (Markham *et al.*, 1998) diluted 1/500 in 1%

BSA/PBS containing 0.05 % Tween 20 (1 % BSA/PBS-T) with gentle rocking, then washed three times as before. The membrane was then incubated for 1 h at room temperature with swine anti-rabbit horseradish peroxidase conjugate (Dako) diluted 1/1000 in 1 % BSA/PBS-T and then washed three times as before. Conjugate binding was visualized using the chromogen 3,3'-diaminobenzidine (Sigma).

Electrophoresis of digested DNA and Southern blotting. Approximately 2 µg mycoplasma DNA was digested to completion with at least 20 U *Eco*RI (Boehringer Mannheim) or *Bgl*II (Boehringer Mannheim) for 2 h at 37 °C in the manufacturer's buffer. The fragments of digested DNA were separated by electrophoresis through 0.7 % agarose (Pharmacia) overnight at 1.25 V cm⁻¹ and blotted onto Hybond-N+ (Amersham) as described previously (Markham *et al.*, 1993). The membrane was incubated at 55 °C for 4 h in Church's prehybridization buffer (Church & Gilbert, 1984), radiolabelled probes were added and hybridized overnight, after which the membrane was washed and autoradiographed using XAR-5 film (Kodak).

DNA probes used in Southern blotting. Radiolabelled probes were prepared from two cloned DNA fragments using [α -³²P]dATP (Amersham) and the Random Primed DNA Labelling kit (Boehringer Mannheim). One of these fragments was a *Pst*I-*Bgl*II DNA fragment (1.2 kbp) containing the amino-terminal region of the pMGA1.2 gene (Markham *et al.*, 1993), and the other a 2.1 kbp cloned PCR product from *M. imitans* (this paper). Both radiolabelled probes were hybridized to the membrane at 55 °C overnight. The membrane probed with the 1.2 kbp fragment was washed three times at 55 °C in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 0.5 % SDS for 5 min each, whilst the membrane probed with the 2.1 kbp fragment was washed three times at 60 °C in 2× SSC, 0.5 % SDS for 10 min each. Two oligonucleotides were used in Southern blotting: Oligo 1 (5'-GAAGAAGAAGAA-GAAGAAGAAGAA-3') and Oligo 2 (5'-ACGAACCAATACCTAATAAACTAACAAA-3') (Baseggio *et al.*, 1996). Each oligonucleotide was 5'-phosphorylated using polynucleotide kinase (Boehringer Mannheim) in the presence of [γ -³²P]ATP (Amersham) and hybridized with the membrane overnight at 50 °C. The membrane was then rinsed twice at room temperature followed by two washes at 50 °C for 10 min each in 6× SSC/0.5 % SDS. The membranes were then autoradiographed using XAR-5 film (Kodak).

PCR, cloning of PCR products and genomic DNA and DNA sequencing. The oligonucleotide T12 (5'-TTTGTAGTTT-ATTAGGTATTGGTTTCG-3'), corresponding to the conserved DNA sequence of the leader peptide of pMGA family members, and the oligonucleotide C13 (5'-CTTCTTCTTCTTCTTCTTCTTCTT-3'), complementary to the conserved trinucleotide repeat sequence (GAA)_n preceding known pMGA genes (Markham *et al.*, 1994), were used as PCR primers. PCR was performed in a 50 µl volume of 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris/HCl (pH 9.0 at 25 °C), with 0.4 mM of each dNTP, 0.4 µM of each primer, approximately 5 ng mycoplasma DNA and 1.25 U of the thermostable *Taq* DNA polymerase (Promega). The PCR was conducted using the Omnigene (Hybaid) thermocycler with an initial incubation at 94 °C for 120 s followed by 30 cycles of 50 °C for 20 s, 74 °C for 90 s and 94 °C for 20 s. PCR products from *M. imitans* were separated by electrophoresis in 1.0 % low-melting-point agarose gel and a band of approximately 2 kbp was excised and purified using the Wizard Prep System (Promega) according to the manufacturer's instructions. Approximately 100 ng of the purified *M. imitans* PCR product

was ligated to the pGEM-T vector (Promega). The ligation mixture was used to transform *E. coli* DH5 α cells and transformants were screened for the presence of pGEM-T carrying a 2 kbp insert. Plasmid DNA was purified using the QIA filter Plasmid Midi Kit purification system (Qiagen). The nucleotide sequence of the 2 kbp insert was determined by the dideoxy chain-termination method initially using oligonucleotides complementary to the vector and subsequently using oligonucleotide primers complementary to determined nucleotide sequence. A genomic library of *Hind*III-digested *M. imitans* genomic DNA in pUC18 was screened using the cloned PCR product radiolabelled with [α -³²P]dATP (Amersham) as described previously (Markham *et al.*, 1993).

Bioinformatics. The SWISS-PROT and GenBank databases and the genomic databases of *M. genitalium* and *M. pneumoniae* were searched using the BLASTN or BLASTX algorithms (Altschul *et al.*, 1990). Alignment of DNA or protein sequences was carried out using the CLUSTAL W algorithm (Thompson *et al.*, 1994).

RESULTS

Detection of pMGA gene sequences in *M. imitans*

The pMGA gene probe bound to multiple bands in Southern blots of *M. gallisepticum* and *M. imitans* genomic DNA digested with either *Eco*RI or *Bgl*II (Fig. 1b). The probe did not bind to genomic DNA of *M. pneumoniae* strains FH, M129 or PI1248, *M. penetrans*, *M. pirum* or *M. genitalium*. Longer exposure (seven times longer than that of Fig. 1b) revealed that up to eight genomic fragments of *M. imitans* and a 1.8 kbp *Eco*RI fragment of *M. iowae* bound the probe.

The Oligo 1 probe, which was complementary to the GAA repeat sequence associated with pMGA genes in *M. gallisepticum*, bound multiple bands in Southern blots of *M. gallisepticum* and *M. imitans* genomic DNA digested with *Eco*RI or *Bgl*II, but did not bind to genomic fragments of *M. pneumoniae*, *M. penetrans*, *M. pirum*, *M. genitalium* or *M. iowae* (Fig. 1c). The patterns of hybridizing fragments of *M. gallisepticum* and *M. imitans* DNA were similar with both the pMGA gene and Oligo 1 probes. A third probe, Oligo 2, which was complementary to the highly conserved leader peptide region of pMGA genes, also bound to a similar group of fragments in a Southern blot of *Eco*RI-digested *M. gallisepticum* genomic DNA and a single 2 kbp fragment of *Eco*RI-digested *M. imitans* genomic DNA (results not shown).

Detection of a protein antigenically related to pMGA in *M. imitans*

Rabbit anti-pMGA antisera reacted with a band of approximately 67 kDa in a Western blot of *M. gallisepticum* proteins (Fig. 2b, lane 7) and with a single band of approximately 35 kDa in *M. imitans* (Fig. 2b, lane 1), but not with any proteins of other *Mycoplasma* species. Monoclonal antibodies mAb66, 71 and 86 (Markham *et al.*, 1992), which are specific for pMGA, did not bind to any protein in Western blots of whole-cell preparations of *M. imitans*, *M. iowae* or *M. pneumoniae*.

Fig. 1. Restriction endonuclease digestion and Southern blotting of mycoplasma DNA. Mycoplasma genomic DNA was digested with *EcoRI* (lanes 1–9 of each panel) or *Bgl/II* (lanes 10–16 of each panel). The resultant fragments were separated by electrophoresis, stained with ethidium bromide (a), then Southern blotted and probed with ^{32}P -radiolabelled 1.2 kb *PstI*–*Bgl/II* DNA fragment (b) or Oligo 2 (c). The *Mycoplasma* species were: 1 and 10, *M. pneumoniae* FH; 2 and 11, *M. pneumoniae* M129; 3 and 12, *M. pneumoniae* PI1428; 4 and 13, *M. penetrans*; 5, *M. pirum*; 6 and 14, *M. genitalium*; 7 and 15, *M. gallisepticum*; 8, *M. iowae*; 9 and 16, *M. imitans*. DNA molecular mass standards were *HindIII*-digested lambda phage DNA.

Fig. 2. SDS-PAGE and immunostaining of mycoplasma cell proteins. Mycoplasma whole-cell proteins were separated by SDS-PAGE and either stained with Coomassie brilliant blue (a) or Western transferred and probed with rabbit antisera raised against pMGA (b). The species were: 1, *M. imitans*; 2, *M. iowae*; 3, *M. pneumoniae* FH; 4, *M. genitalium*; 5, *M. penetrans*; 6, *M. pirum*; 7, *M. gallisepticum*. Molecular mass standards were: phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa (Pharmacia Biotech).

Cloning and sequencing of a gene from *M. imitans* exhibiting similarity to members of the *M. gallisepticum* pMGA gene family

As oligonucleotide probes Oligo 1 and 2 hybridized with *M. imitans* genomic DNA, PCR was conducted using a complementary primer set (T12 and C13). When used in PCR with *M. gallisepticum* genomic DNA as template,

these primers are predicted to amplify fragments of approximately 2.2 kbp with some variation in size depending on which pMGA genes were amplified. The architecture of pMGA genes meant that these fragments would contain a truncated pMGA gene which began within the coding sequence for the leader peptide, included the coding sequence for the mature peptide and ended with the GAA trinucleotide repeat of the inter-

Fig. 3. Alignment of the pMGA1.1 and *M. imitans* VlhA1 and VlhA2 partial amino acid sequences. The three amino acid sequences were aligned and gaps were introduced within the sequence to maximize identity. Identical residues between sequences are shaded black and the signal peptidase II cleavage site is overlined.

genic region preceding the next pMGA gene. Using *M. imitans* genomic DNA as the template in PCR, two major products of 800 bp and 2100 bp were amplified (results not shown), while use of *M. gallisepticum* DNA as the template resulted in products of 800 bp and 2200 bp (results not shown). No products were detected when *M. pneumoniae* DNA was used as the PCR template.

The larger PCR product from *M. imitans* was purified from the agarose gel, cloned into the pGEM-T vector (Promega) and subjected to DNA sequencing. The size of the PCR product, including primers, was 2.1 kbp. The 2.1 kbp cloned PCR product was then used to screen a *Hind*III-digested library of *M. imitans* genomic DNA. The plasmid contained by this isolate contained a 3.1 kbp insert, the sequence of which overlapped the cloned PCR fragment. The two sequences were identical throughout the overlapping region and together covered 3.331 kbp of contiguous sequence containing one com-

plete ORF (*vlhA1*) and a second partial ORF terminating at a *Hind*III cleavage site (*vlhA2*).

Comparison of both ORFs with SWISS-PROT and GenBank databases revealed that *M. gallisepticum* pMGA DNA and protein sequences had significant identity with the cloned *M. imitans* DNA and both predicted amino acid sequences. The organization of the *M. imitans* genes was similar to that of *M. gallisepticum* pMGA genes, with the GAA trinucleotide repeats commencing 169 nucleotides downstream of the stop codon of the previous gene. Translation of the first ORF, followed by post-translational cleavage of the leader peptide (see below), would yield a mature protein of 616 aa with a molecular mass of 65 285 Da. The amino acid sequences of pMGA1.1, VlhA1 and VlhA2 were aligned for maximum identity (Fig. 3), producing a consensus sequence showing amino acids in common. The three sequences included the amino acid sequence V/A A S C, which preceded the amino-terminal cysteine and con-

Fig. 4. Southern blot of mycoplasma genomic DNA probed with an *M. imitans* 2.1 kbp DNA fragment. Mycoplasma genomic DNA was digested with *Eco*RI (lanes 1–9) or *Bgl*II (lanes 10–16). The *Mycoplasma* species were: 1 and 10, *M. pneumoniae* FH; 2 and 11, *M. pneumoniae* M129; 3 and 12, *M. pneumoniae* PI1428; 4 and 13, *M. penetrans*; 5, *M. pirum*; 6 and 14, *M. genitalium*; 7 and 15, *M. gallisepticum*; 8, *M. iowae*; 9 and 16, *M. imitans*. DNA molecular mass standards were *Hind*III-digested lambda phage DNA.

formed to the consensus sequence for cleavage by signal peptidase II, and proline repeat sequences in the amino-terminal region of the mature peptide. Several stretches of amino acid sequence that were shared by all predicted pMGA polypeptides were also conserved in the *M. imitans* sequence.

The cloned 2.1 kbp PCR product of *M. imitans* DNA contains a member of a gene family

To verify that the cloned PCR fragment was derived from *M. imitans* genomic DNA the 2.1 kbp insert was excised from the pGEM-T vector, radiolabelled with 32 P and used to probe a Southern blot of genomic DNA of different *Mycoplasma* species. The Southern blot was washed under moderately stringent conditions and autoradiographed. The 2.1 kbp probe hybridized most strongly to a 4.5 kbp fragment in *Eco*RI-digested *M. imitans* DNA and fragments of 13 kbp and 2.3 kbp in *Bgl*II-digested *M. imitans* DNA (Fig. 4, lanes 9 and 16, respectively) and also bound a 3.3 kbp fragment in *Hind*III-digested *M. imitans* DNA (results not shown). A total of 42 kbp of *M. imitans* genomic DNA fragments bound to the 2.1 kbp probe and the pattern of fragments bound was identical to that of fragments bound by Oligo 1 and the pMGA DNA fragment.

The 2.1 kbp probe also bound the same fragments of *M.*

gallisepticum DNA (Fig. 4, lanes 7 and 15) as those bound by the 1.2 kbp pMGA probe and the Oligo 1 probe (Fig. 1b and c, respectively). The 2.1 kbp probe also bound a 1.8 kbp *Eco*RI fragment of *M. iowae* genomic DNA (Fig. 4, lane 8).

There was weak hybridization of the *M. imitans* probe to *M. genitalium* genomic DNA with a fragment of 13 kbp bound in *Eco*RI digests and fragments of 8.6 kbp and 7.2 kbp bound in *Bgl*II digests (Fig. 4, lanes 6 and 14). The *M. genitalium* database was searched for similarity with the 2.1 kbp sequence using the BLAST search algorithm (Altschul *et al.*, 1990) and several sequences were detected with varying levels of DNA identity. The segment of the *M. genitalium* genome (U39715) which had the highest similarity scores contained a GAA repeat trinucleotide and would yield *Eco*RI and *Bgl*II fragments of 13 kbp and 8 kbp, respectively, similar in size to the observed weakly hybridizing fragments. However this region did not contain an ORF homologous to the pMGA genes.

Conservation of 5' non-coding regions on either side of the transcriptional control motif

Alignment of the 5' non-coding region of all characterized pMGA genes and of the *vlbA2* gene characterized in this study revealed considerable conservation of sequences between the GAA repeat motif known to control transcription of pMGA genes and the GTG start codon (Fig. 5). There were several regions of very high sequence conservation across all genes, notably at the –10 region (GAAAAT), at the transcriptional start point and the seven bases 3' to it (GATTTATT), and just 5' to the GTG start codon (TTTAA). In addition there were several highly conserved sequences 5' to the GAA repeat motif and a highly conserved region about 60 bases 5' to the start codon.

DISCUSSION

This study has identified and characterized a gene family in *M. imitans* that is homologous to lipoprotein gene families in *M. gallisepticum* (the pMGA gene family) and *M. synoviae* (the *vlbA* gene family). It seems appropriate that the three families be recognized as homologous by referring to them all as *vlbA* gene families.

The pMGA genes of *M. gallisepticum* exhibit varying levels of DNA identity (40–98%) and the coding regions share several structural features including a 25-amino-acid leader sequence and signal peptidase II consensus cleavage sequence (Markham *et al.*, 1993, 1994; Baseggio *et al.*, 1996). The family members are predominantly organized as tandem repeats with an intergenic region of 350 bp. A consistent feature of the intergenic region is a GAA trinucleotide repeat region immediately preceding the promoter which has recently been shown to control transcription of these genes (Glew *et al.*, 1998). The two genes characterized in *M.*

Fig. 5. Alignment of part of the 5' non-coding region of pMGA genes of *M. gallisepticum* and the *vlhA2* gene of *M. imitans*. Gaps (shown as dots) were introduced within the sequence to maximize identity, dashes indicate bases conserved in all eight sequences and bases are shown if different from the consensus. Conserved regions are apparent either side of the GAA repeat region known to be responsible for control of transcription in the pMGA genes. The predicted -10 region is indicated together with the transcriptional start site (+1) and start codon.

imitans possessed a typical signal peptidase II cleavage sequence, a proline-rich imperfect repeat region at the amino-terminus of the mature predicted lipoprotein and several other regions of high sequence similarity which coincided with the most conserved regions of the pMGA genes. In addition one of the genes was found to be part of a gene family in *M. imitans*, the distribution of which had a close concordance with the distribution of GAA repeat sequences in the *M. imitans* genome. The GAA repeat region was located a similar distance 3' to the end of coding sequence of the gene, compared to its location in pMGA genes, suggesting that the *M. imitans* gene family was also organized as tandem repeats, and that it was also likely to be regulated in a similar manner to the pMGA family. With up to 42 kbp of genomic DNA hybridizing to the probe for the *M. imitans* gene, there may be up to 19 family members, assuming the gene length is 2.2 kbp, as seen in the genes characterized in this study. It should be recognized that this is likely to be an overestimate of the extent of the family as it assumes that all hybridizing fragments are entirely composed of full-length copies of members of the family.

In Southern blot analysis the cloned 2.1 kbp fragment of *M. imitans* bound most strongly to a number of *M. imitans* and *M. gallisepticum* genomic DNA fragments and a single 1.8 kbp *EcoRI* fragment of *M. iowae*. The pMGA1.2 DNA probe also bound the 1.8 kbp *EcoRI* fragment of *M. iowae*, suggesting DNA sequences in common with both DNA probes. Faint reactivity was also observed between the 2.1 kbp probe and *M. genitalium* DNA, but a search of the *M. genitalium*

genomic database suggested that this was associated with a limited GAA trinucleotide repeat region, not with homologous coding sequences.

Homologues of the *vlhA* families were not detected in other members of the pneumoniae phylogenetic group. However, it should be noted that the nucleic acid identity between pMGA genes and the *M. imitans* genes was between 49.7 % and 60.2 % and, as *M. imitans* and *M. gallisepticum* are much more closely related to each other than any other *Mycoplasma* species, homologues in other species may be difficult to identify using the methods applied in this study unless they were also associated with the characteristic GAA trinucleotide repeat.

Searches of the *M. pneumoniae* and *M. genitalium* databases with either the complete peptide sequences or consensus sequences or shorter conserved regions of pMGA genes and the *M. imitans* homologue failed to identify homologous genes. There are no GAA trinucleotide repeat regions in either genome which are located near predicted lipoprotein genes. While these findings suggest the absence of pMGA homologues in *M. pneumoniae* and *M. genitalium*, it is notable that the *M. pneumoniae* genome contains six lipoprotein gene families, none of which have identifiable homologues in any other bacterial species, and most members of these families are, like the pMGA genes, organized as tandem repeats. Further examination of the structure and function of these families may reveal homologues that are not apparent at the level of primary sequence.

Rabbit immune sera raised to pMGA1.1 reacted with a 35 kDa protein of *M. imitans*, which is smaller than the predicted size of the protein encoded by the ORF characterized in this study. This could be due to another *vlhA* gene family member encoding a smaller product or possibly result from post-translational cleavage of the gene product. The product of *vlhA* in *M. synoviae* is usually completely cleaved into an amino-terminal lipoprotein and a carboxyl-terminal haemagglutinin, and post-translational cleavage has been observed for one of the pMGA gene products in *M. gallisepticum*, although in this case some of the product remains uncleaved (Noormohammadi *et al.*, 1997, 1998; Markham *et al.*, 1998). Further studies will need to be conducted to determine if either of these propositions is correct.

Areas of conservation of the 5' non-coding sequence of these genes, both within and between species, may provide further information about factors involved in the control of transcription and translation, both areas poorly understood in mycoplasmas. The high level of conservation of the GAAAAT motif 10 bases 5' to the transcriptional start point suggests that this represents the -10 box, although the best consensus with *E. coli* promoters would be several bases 3' to this. The other conserved regions could be hypothesized to play regulatory roles in either transcription or translation. Most importantly conservation 5' to the GAA repeat motif known to control transcription lends support to the hypothesis that the length of this motif controls transcription by providing optimal spacing between RNA polymerase and a factor binding 5' to the repeat motif (Glew *et al.*, 1998). Experimental approaches to investigation of mycoplasma promoters are limited at present, and such observations provide a better focus for future studies of transcriptional control.

The *vlhA* gene from *M. synoviae* encodes a surface-exposed lipoprotein that shares 41% amino acid sequence similarity and 24% amino acid identity with VlhA1, indicating that VlhA1 is more closely related to *M. gallisepticum* pMGA1.1 and related sequences. It has been suggested that the gene families shared by *M. synoviae* and *M. gallisepticum* may have arisen by horizontal gene transfer, as pMGA gene homologues could not be identified in *M. pneumoniae* or *M. genitalium*, both of which are phylogenetically closer to *M. gallisepticum* than *M. synoviae* (Noormohammadi *et al.*, 1998). Since *M. gallisepticum*, *M. synoviae* and *M. imitans* infect gallinaceous species, the observation that the same gene family occurs in each of these avian mycoplasmas but not in phylogenetically related human mycoplasmas strengthens the speculation that this family has been transferred horizontally between mycoplasmas sharing avian hosts. Curiously, the *vlhA* family of *M. synoviae* is not associated with GAA trinucleotide repeats, suggesting that, if the gene family originated from *M. synoviae*, the transfer preceded the divergence of *M. gallisepticum* and *M. imitans*, and that the coding regions and the promoter regions of the gene family have independent origins.

ACKNOWLEDGEMENTS

The authors wish to thank Associate Professor I. D. Walker for his helpful comments in the preparation of this manuscript. This research was funded by grants from the Australian Research Council and Bioproperties (Australia) Pty Ltd.

REFERENCES

- Abdul, W. O., Ross, G. & Bradbury, J. M. (1996). Pathogenicity and cytoadherence of *Mycoplasma imitans* in chicken and duck embryo tracheal organ cultures. *Infect Immun* **64**, 563–568.
- Altschul, S. F., Gish, W., Miller, W. & Myers, E. W. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Baseggio, N., Glew, M. D., Markham, P. F., Whithear, K. G. & Browning, G. F. (1996). Size and genomic location of the pMGA multigene family of *Mycoplasma gallisepticum*. *Microbiology* **142**, 1429–1435.
- Behbahani, N., Blanchard, A., Cassell, G. H. & Montagnier, L. (1993). Phylogenetic analysis of *Mycoplasma penetrans*, isolated from HIV-infected patients. *FEMS Microbiol Lett* **109**, 63–66.
- Boyle, J. S. & Morrow, C. J. (1994). Phylogeny of avian mycoplasmas: implications for mollicute taxonomy. *Int Org Mycoplasmol Lett* **3**, 589.
- Boyle, J. S., Good, R. T. & Morrow, C. J. (1995). Detection of the turkey pathogens *Mycoplasma meleagridis* and *M. iowae* by amplification of genes coding for rRNA. *J Clin Microbiol* **33**, 1335–1338.
- Bradbury, J. M., Abdul, W. O., Yavari, C. A., Dupiellet, J. P. & Bove, J. M. (1993). *Mycoplasma imitans* sp. nov. is related to *Mycoplasma gallisepticum* and found in birds. *Int J Syst Bacteriol* **43**, 721–728.
- Church, G. M. & Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci USA* **81**, 1991–1995.
- Dallo, S. F. & Baseman, J. B. (1990). Cross-hybridization between the cytoadhesin genes of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* and genomic DNA of *Mycoplasma gallisepticum*. *Microb Pathog* **8**, 371–375.
- Ganapathy, K. & Bradbury, J. M. (1998). Pathogenicity of *Mycoplasma gallisepticum* and *Mycoplasma imitans* in red-legged partridges (*Alectoris rufa*). *Avian Pathol* **27**, 455–463.
- Ganapathy, K., Jones, R. C. & Bradbury, J. M. (1996). Pathogenicity of *in vivo*-passaged *Mycoplasma imitans* in turkey poults in single infection and in dual infection with infectious bronchitis virus. *Avian Pathol* **27**, 80–89.
- Glew, M. D., Baseggio, N., Markham, P. F., Browning, G. F. & Walker, I. D. (1998). Expression of the pMGA genes of *Mycoplasma gallisepticum* is controlled by variation in the GAA trinucleotide repeat lengths within the 5' noncoding regions. *Infect Immun* **66**, 5833–5841.
- Goh, M. S., Gorton, T. S., Forsyth, M. H., Troy, K. E. & Geary, S. J. (1998). Molecular and biochemical analysis of a 105 kDa *Mycoplasma gallisepticum* cytoadhesin (GapA). *Microbiology* **144**, 2971–2978.
- Inamine, J. M., Loechel, S., Collier, A. M., Barile, M. F. & Hu, P.-C. (1989). Nucleotide sequence of the *MgPa* (*mgp*) operon of *Mycoplasma genitalium* and comparison to the *P1* (*mpp*) operon of *Mycoplasma pneumoniae*. *Gene* **82**, 259–267.
- Keeler, C. L., Hnatow, L. L., Whetzel, P. L. & Dohms, J. E. (1996). Cloning and characterization of a putative cytoadhesin gene (*mgc1*) from *Mycoplasma gallisepticum*. *Infect Immun* **64**, 1541–1547.
- Kleven, S. H., Browning, G. F., Bulach, D. M., Ghiocas, E., Morrow,

- C. J. & Whithear, K. G. (1988). Examination of *Mycoplasma gallisepticum* strains using restriction endonuclease DNA analysis and DNA-DNA hybridisation. *Avian Pathol* 17, 559–570.
- Markham, P. F., Glew, M. D., Brandon, M. R., Walker, I. D. & Whithear, K. G. (1992). Characterization of a major hemagglutinin protein from *Mycoplasma gallisepticum*. *Infect Immun* 60, 3885–3891.
- Markham, P. F., Glew, M. D., Whithear, K. G. & Walker, I. D. (1993). Molecular cloning of a member of the gene family that encodes pMGA, a hemagglutinin of *Mycoplasma gallisepticum*. *Infect Immun* 61, 903–909.
- Markham, P. F., Glew, M. D., Sykes, J. E., Bowden, T. R., Pollocks, T. D., Browning, G. F., Whithear, K. G. & Walker, I. D. (1994). The organisation of the multigene family which encodes the major cell surface protein, pMGA, of *Mycoplasma gallisepticum*. *FEBS Lett* 352, 347–352.
- Markham, P. F., Glew, M. D., Browning, G. F., Whithear, K. G. & Walker, I. D. (1998). The expression of two members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is influenced by pMGA-specific antibodies. *Infect Immun* 66, 2845–2853.
- Noormohammadi, A. H., Markham, P. F., Whithear, K. G., Walker, I. D., Gurevich, V. A., Ley, D. H. & Browning, G. F. (1997). *Mycoplasma synoviae* has two distinct phase-variable major membrane antigens, one of which is a putative hemagglutinin. *Infect Immun* 65, 2542–2547.
- Noormohammadi, A. H., Markham, P. F., Duffy, M. F., Whithear, K. G. & Browning, G. F. (1998). Multigene families encoding the major hemagglutinins in phylogenetically distinct mycoplasmas. *Infect Immun* 66, 3470–3475.
- Su, C. J., Chavoya, J. & Baseman, J. B. (1988). Regions of *Mycoplasma pneumoniae* cytoadhesin P1 structural gene exist as multiple copies. *Infect Immun* 56, 3157–3161.
- Tham, T. N., Ferris, S., Bahraoui, E., Canarelli, S., Montagnier, L. & Blanchard, A. (1994). Molecular characterization of the P1-like adhesin gene from *Mycoplasma pirum*. *J Bacteriol* 176, 781–788.
- Thompson, J. D., Higgins, D. G. & Gibson, T. G. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22, 4673–4680.
- Weisburg, W. G., Tully, J. G., Rose, D. L. & 9 other authors (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* 171, 6455–6467.

Received 17 February 1999; revised 26 April 1999; accepted 13 May 1999.