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The organisation of the multigene family which encodes the major cell surface protein, pMGA, of *Mycoplasma gallisepticum*

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Abstract The genome of the avian pathogen *Mycoplasma gallisepticum* contains a number of related genes for putative adhesion molecules (pMGA). Cloning and sequence analysis of several pMGA genes suggested that all of them might be transcriptionally and translationally functional. Analysis of the gene sequence encoding the sole pMGA variant expressed in vitro in the S6 strain (pMGA1.1) revealed no unambiguous feature that could account for its unique expression. It is estimated that the pMGA gene family may contain up to 50 members, and its possible role is discussed herein.

Key words: *Mycoplasma*; Adhesin; Promoter; Gene expression; Sequence variability

1. Introduction

We recently described a major surface protein of the avian pathogen *Mycoplasma gallisepticum* (pMGA) which may play a role in the adhesion between host cells and *M. gallisepticum* cells [1]. The M_r of the pMGA protein is approximately 67,000 and it lacks a hydrophobic peptide region which might act as a membrane anchor. It is likely instead to be attached to the *M. gallisepticum* plasma membrane via a lipid anchor attached to its amino-terminal cysteine residue [1,2]. The pMGA molecules from three independent clonal isolates of *M. gallisepticum* differed in M_r and/or in their expression of an epitope, defined by a monoclonal antibody directed to the pMGA molecule expressed by the S6 strain of *M. gallisepticum*. Molecular cloning of the DNA of a single strain of *M. gallisepticum* (the S6 strain) resulted in the isolation and partial characterisation of two genes termed pMGA1.2 and pMGA1.3, each encoding a pMGA-like protein. However, neither gene encoded exactly the pMGA protein expressed by cells of the S6 strain (pMGA1.1) although the pMGA1.2 gene, in particular, could encode a polypeptide variant which was more than 90% identical to the pMGA protein expressed by this strain. The pMGA1.2 and pMGA1.3 genes were in the same transcriptional orientation and were separated by 434 non-coding nucleotides. Both genes appear potentially functional as their inferred coding sequences lack premature stop codons. In this paper we report the results of studies to determine how many pMGA-like genes the *M. gallisepticum* genome might contain, to examine the arrangement of these genes within the genome, and to isolate and characterise the gene which encodes the pMGA molecule expressed in the S6 strain of *M. gallisepticum*.

2. Experimental

2.1. Bacterial strains, cultural methods and preparation of DNA

The S6, R and F strains of *M. gallisepticum*, which have been used in previous work [1], were also used for this study. DNA was extracted from these strains for molecular cloning and Southern blot hybridization as described previously [2]. Briefly, cells were grown in mycoplasma broth medium supplemented with 10% swine serum until late log phase and harvested by centrifugation at 20,000 × *g*. Cells were washed twice in 0.1 M phosphate buffer, 0.33 M NaCl, pH 7.4 (NaOH), and lysed in 10 mM Tris, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, pH 8.0 (HCl). The lysate was digested with 100 μg/ml of proteinase K at 37°C overnight, then with 1 mg/ml of RNase A at 37°C for 30 min. This digested lysate was extracted with phenol, phenol:chloroform:isoamylalcohol (25:24:1), chloroform:isoamylalcohol (24:1), and the DNA finally precipitated with ethanol.

2.2. Molecular cloning of pMGA-like genes

An *Eco*RI fragment of DNA of approximately 10 kilobases (kb) from the *M. gallisepticum* S6 genome was cloned and partially sequenced previously [2]. The remainder of this fragment was sequenced in this study. In our previous study a second *Eco*RI fragment, 6 kb in length, was identified as reactive with two oligonucleotides designed to be complementary to the coding sequence for peptide fragments of the pMGA molecule. This fragment was cloned within a larger *Cla*I fragment as follows. Genomic DNA was digested to completion with the restriction endonuclease *Cla*I. This DNA was ligated into *Cla*I-digested pBluescript SK$^-$, and the product used to transform *E. coli* strain DH5α by electroporation. Clones were chosen for further characterisation based on the ability of the 10 kb *Eco*RI fragment to hybridize to colony blots of their DNA. A clone containing a 12 kb *Cla*I fragment was found to contain a 6 kb *Eco*RI fragment which hybridized strongly to the 10 kb fragment, and to fragments containing the pMGA1.2 gene. The *Pst*I and two *Pst*I-*Eco*RI subclones from this fragment were selected for DNA sequencing.

2.3. Southern blotting

Southern blotting was performed essentially as described previously [2]. Briefly genomic DNA from S6, R and F strains was digested to completion with the restriction endonucleases, *Eco*RI, *Hin*dIII and *Bgl*II. Complete digestion of genomic DNA was verified by comparison of ethidium bromide profiles of genomic DNA digested using half the number of units of enzyme. The fragments were separated by electrophoresis in 0.5% agarose gels, and transferred to nylon membranes. Two ^{32}P-labelled probes prepared from subclones of the 9.72 kb *Eco*RI clone ((i) a *Pst*I/*Bgl*II fragment containing the major portion of the pMGA1.2 nt sequence (Fig. 1A); and (ii) a *Bgl*II/*Bgl*II fragment containing part of the pMGA1.2-intergenic-pMGA1.3 nt sequence (Fig. 1A)), were seperately hybridized to these blots at 50°C in 6 × SSC and washed three times in 1 × SSC, 0.5% SDS.

2.4. DNA sequencing using exonuclease III truncations

Purified circular plasmid DNA was digested with *Pst*I (Boehringer-Mannheim) and *Sal*I (Amersham) restriction enzymes according to the manufacturer's recommendations. Exonuclease III (Amersham) digestions were conducted at 35°C and small samples were withdrawn for storage and analysis every 30 s. These samples were immediately digested with Mung bean nuclease (Amersham), aliquots subjected to

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electrophoretic analysis and at selected time points were then ligated using T4 DNA ligase (Boehringer-Mannheim). A portion of each ligation mix was used to transform competent DH5α *E. coli* cells. Plasmid DNA from individual recombinants was then purified and overlapping clones were subjected to dideoxy sequencing as previously described [2].

3. Results

3.1. The pMGA1.2–1.6 gene cluster

The complete DNA sequence of the cloned 9,720 bp *Eco*RI fragment of *M. gallisepticum* DNA containing the genes for pMGA1.2 and pMGA1.3 [2] was determined. The entire 9.720 kb of DNA was found to consist of tandem repeats of pMGA-like genes separated by 307–434 bp, with all coding sequences in the same transcriptional orientation, and is diagramatically presented in Fig. 1A. The Genbank accession numbers for the nucleotide sequences on which the data of Fig. 1C are derived are L28423 and L28424. The coding sequence of pMGA1.2 (without its leader sequence) would produce a polypeptide of 625 amino acids, a product of similar size to the experimentally measured M_r value (67,000) for *M. gallisepticum* strain S6 pMGA. The mature coding sequences for the two other complete pMGA-like sequences would encode slightly larger polypeptides of around 680 amino acid residues with molecular weights close to 73 kDa. The amino acid sequences of pMGA genes were aligned to maximise sequence similarity using CLUSTAL V [3] and are presented in Fig. 1C. The absence of internal stop codons within all pMGA-like coding sequences is evident from the amino acid translations of Fig. 1C in which the alignment of full (pMGA1.2, 1.3 and 1.4) or partial (pMGA1.5 and 1.6) amino acid sequences of the genes are shown. All these genes occur in the same transcriptional orientation and genes pMGA1.2–1.5 are equipped with functional signal sequences where conservation is particularly high between genes, and with stop codons located at homologous sites. The absence of internal stop codons within any of these genes implies that their protein products are under strong selection to maintain full translational competence.

The sequence alignment of Fig. 1C indicates that no extended region within the pMGA sequences, other than the leader sequence, exhibits notably high conservation. None of the predicted sequences of pMGA1.2–1.6 in Fig. 1C could encode all of the peptide sequences previously reported by us [2], which

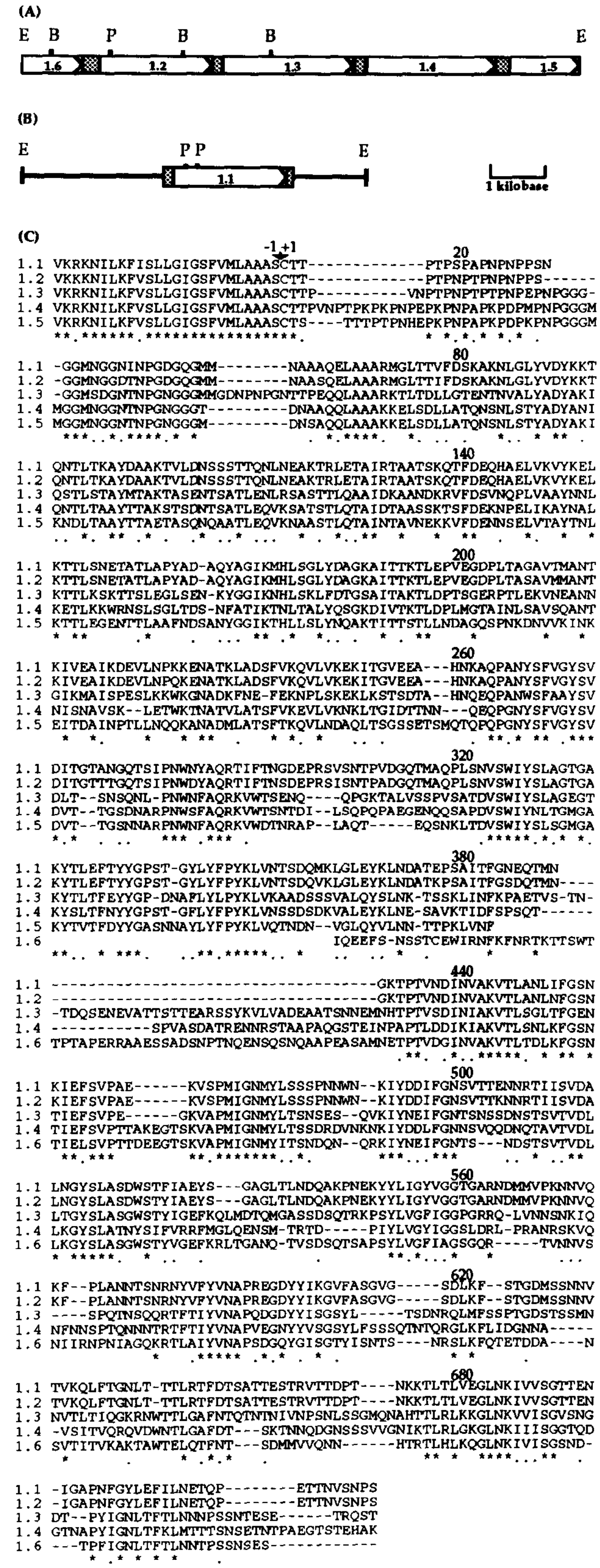

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Fig. 1. The gene organisation and amino acid sequences of pMGA genes 1.1–1.6. The organisation and positioning of pMGA genes: (A) pMGA1.2–1.6 coded for within an 9.72 kb *Eco*RI fragment, (B) pMGA1.1 coded for within an 6 kb *Eco*RI fragment. For A and B the intergenic regions between consecutive pMGA genes are depicted by a shaded rectangle. The transcriptional direction is indicated by an arrow together with the assigned pMGA gene number in the unshaded rectangle. Endonuclease restriction sites used in subcloning are shown: E, *Eco*RI; B, *Bgl*II; P, *Pst*I. (C) The amino acid sequences of pMGA coding sequences, derived from corresponding nucleotide sequences (Genbank accession numbers L28423 and L28424) are aligned to maximise overall mutual homology. Amino acid residues conserved in all sequences are indicated by asterisks, and dots show positions where conservative substitutions of amino acids have occurred. Gaps have been introduced to maximise sequence similarity. The junction between the end of the leader sequences and the start of the mature sequences is indicated by a bold arrowhead and residues are numbered from the (putative) amino-terminal cysteine residue of all sequences.

were derived from the pMGA protein of *M. gallisepticum* strain S6: the pMGA1.2–1.6 genes are thus not expressed in this strain during in vitro culture, at least at the protein level (see section 4).

3.2. The pMGA1.1 gene

It was of obvious interest to clone and characterise the gene expressed in the S6 strain of *M. gallisepticum*. A 9.720 kb fragment (Fig. 1A) and a distinct 6 kb fragment had been uniquely detected previously in *Eco*RI-digested *M. gallisepticum* genomic DNA by two oligonucleotide probes based on peptide sequences of the strain S6 pMGA molecule [2]. Since the open reading frames of the 9.72 kb fragment do not encode the pMGA protein expressed by S6 in vitro, it was most likely that the 6 kb fragment would contain the gene encoding this pMGA molecule and the 6 kb fragment was therefore cloned and partially sequenced. The predicted amino acid sequence of a particular pMGA gene within the 6 kb fragment concorded almost perfectly with all of the previously established peptide sequences of pMGA. No other segment of the 6 kb cloned fragment, or indeed of any other *M. gallisepticum* genomic fragment, other than the 9.720 kb fragments exhibited reactivity to the two oligonucleotides based on the pMGA protein sequence. The gene is listed as pMGA1.1 (so called to denote its capacity to encode the S6 strain pMGA protein), the gene position within the 6 kb *Eco*RI-cloned fragment is shown in Fig. 1B and the complete amino acid sequence is shown in Fig. 1C. Its predicted sequence exhibits homology to the other pMGA sequences determined in this work (identities between all pMGA sequences are indicated by asterisks in Fig. 1C). 234 out of 650 amino acids within the predicted pMGA1.1 sequence (including the leader sequence) concord with the pMGA consensus sequence. The amino acid identity between pMGA1.1 and pMGA1.2 sequences is 96% and there is only one amino acid discrepancy between the pMGA1.1 sequence and our previously reported pMGA peptide sequences. This difference, an Asn→Thr substitution, may be due to cloning of DNA from a minor variant with a single base substitution or to mutation during propagation of the cloned fragment. Based on the Southern transfer analysis using oligonucleotides based on the pMGA protein expressed by strain S6 as probes, there is no other *M. gallisepticum* genomic fragment which could contain an alternative expressed gene.

3.3. Divergence of pMGA gene structures between Mycoplasma gallisepticum strains

To assess the degree of sequence divergence within the pMGA gene clusters of different *M. gallisepticum* strains, DNA samples from three different well-characterised strains, S6, R and F, were digested with restriction endonucleases and exam-

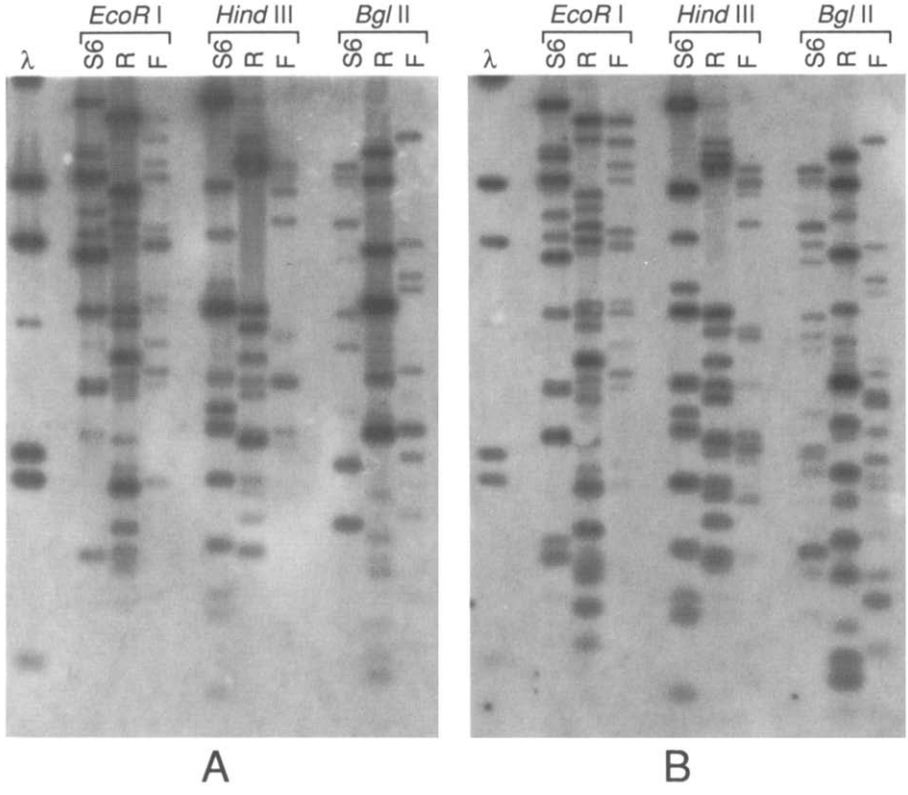

Fig. 2. Restriction enzyme digests of three strains of *M. gallisepticum*. DNA samples from three strains of *M. gallisepticum*, S6, R and F, were digested with the restriction enzymes shown and subjected to Southern blot analysis. Panel A was obtained using a labelled probe corresponding to the *PstI*/*BglII* fragment containing the major portion of the pMGA1.2 nt sequence (Fig. 1A); panel B was obtained using a contiguous, non-overlapping *BglII*/*BglII* fragment containing part of the pMGA1.2-intergenic-pMGA1.3 nt sequence (Fig. 1A) as probe. Agarose gels (0.5%) were used to conduct electrophoresis in all cases and channels containing molecular weight markers (*Hin*dIII digest of λ DNA) are indicated (λ), with fragments corresponding to 23.1, 9.6, 6.7, 4.4, 2.3, 2.1 and 0.56 kb. Labelling of the molecular weight markers was achieved by including radiolabelled λ DNA in hybridizations. Control experiments have established that the λ DNA probe does not hybridize to the *M. gallisepticum* DNA under the conditions used.

ined using Southern blot analysis. The probes used in this analysis were derived from non-overlapping segments of the pMGA1.2 and 1.3 genes as described in section 2.

The patterns obtained in these experiments revealed that multiple DNA fragments hybridized to both labelled probes in all DNA samples examined (Fig. 2). Notably, similar or identical patterns were obtained with both probes with DNA samples from the same strain digested with the same restriction enzyme, albeit with some differences between the two probes in the relative intensity of binding to corresponding fragments. Marked differences were apparent between strains. In fact no major DNA fragment was found common to all three strains, and few fragments were common between any two strains. The stringency used to conduct the hybridization reactions involved in the experiment of Fig. 2 was relatively low, and this accounts for the complexity of the observed patterns relative to previous Southern blot experiments from this laboratory [2] in which very high stringencies were used and in which, consequently, only two or three genomic fragments bound these probes.

3.4. The pMGA intergenic regions

The montage of Fig. 3 shows an alignment of the sequences between each of the pMGA genes sequenced in this study. The lengths of these intergenic regions are somewhat variable; the pMGA1.3 segment contains 434 nucleotides between the final TAG stop codon (of pMGA1.2) and the GTG start codon (of pMGA1.3). Other intergenic regions are somewhat shorter than this and the pMGA1.2 intergenic region contains 342 nucleotides. Several general features are apparent from Fig. 3. The first 250 or so nucleotides at the 5′ end of the intergenic alignment exhibit considerable sequence variation. Gaps are frequent as well as single nucleotide substitutions. This segment is followed by a variable number of conserved GAA repeats – 15 in pMGA1.3, 14 in pMGA1.4, 12 in pMGA1.1, 10 in both pMGA1.2 and 1.5. The next segment is highly conserved between intergenic regions and extends for 130–132 nucleotides through to the GTG start codon of all pMGA-like genes.

Sequence motifs which may correspond to promoters are indicated in Fig. 3. Specifically a putative −10 region sequence TATATA (boxed in dotted lines in Fig. 3), resembles the consensus −10 promoter sequence of *E. coli* (TATAAT), and the sequence CCGATT (also boxed in dotted lines) possibly corresponds to the −35 consensus sequence of *E. coli* promoters (TTGACA) [4]. The spacer region between these motifs is 17–18 nucleotides in all pMGA-like genes examined, similar in length to the spacer region of *E. coli* promoters. Another possible set of −10, −35 motifs occurs 5′ to the boxed set in Fig. 3 (solid lines). The possible role of the elements identified within the intergenic regions of pMGA genes is considered in the discussion.

4. Discussion

4.1. The salient features of members of the pMGA family

The predicted amino acid sequences of pMGA1.2–1.6 genes and of pMGA1.1 are listed in Fig. 1C: it is apparent that all pMGA-like genes have evolved from a common ancestor by a series of gene duplications. Certain genes are much more closely related to one another than to other members of the family. For example at the amino acid level, sequence identity between pMGA1.1 and 1.2 is 96% whereas that between

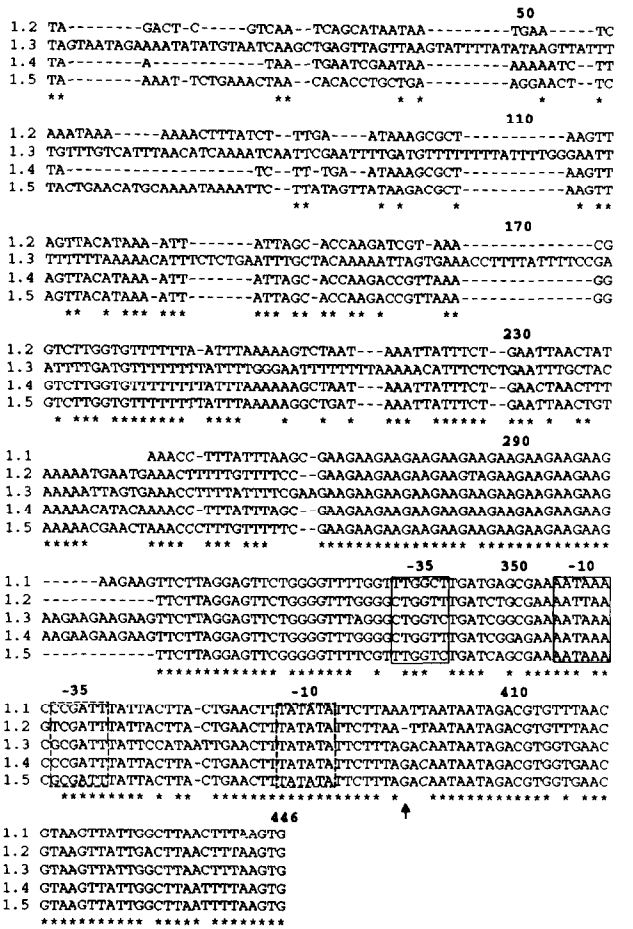

Fig. 3. Sequence alignments of pMGA intergenic regions. The DNA segment 5′ to the pMGA genes indicated are aligned to maximise sequence identities. Each sequence extends from the presumptive stop codon of the preceding pMGA gene to the start (GTG) codon of the indicated gene. Asterisks indicate invariant nucleotides. Putative −10 and −35 promoter regions are boxed and the arrow indicates a possible transcription start site of the pMGA1.1 gene.

pMGA1.1 and pMGA1.3 is around 42%. In the absence of a detailed structural model for pMGA proteins it is impossible to assess the significance of individual amino acid interchanges.

Analysis of the 9.720 kb DNA fragment containing pMGA1.2–1.6 genes revealed that all these genes were tandemly arranged in the same transcriptional orientation and separated by an average of 360 non-coding nucleotides. Preliminary DNA sequencing and analysis of DNA regions adjacent to pMGA1.1 indicates that 3′ to pMGA1.1 is an intergenic region of 353 nt, followed by a partial open reading frame that would encode a member of the pMGA family in the same transcriptional orientation as pMGA1.1 (data not shown). Using oligonucleotide primers to conserved intergenic regions, a further partial DNA sequence upstream to pMGA1.1 was found that would encode a member of the pMGA gene family, however, the transciptional orientation of this sequence could not be confirmed (data not shown).

Attempts to find a consistent reading frame within the pMGA intergenic segments which might demonstrate additional coding potential were unsuccessful (data not shown). The

lengths of the intergenic DNA segments argue against the transcription of a polycistronic RNA containing multiple pMGA-like cistrons. If this were so then only a few nucleotides need be expected between the stop codons and start codons of consecutive coding sequences. In this respect the pMGA system of *M. gallisepticum* differs substantially from the P1 system of the phylogenetically related pathogen *M. pneumoniae* in which the P1 gene is encoded by a polystronic RNA which encodes two additional polypeptides [5]. Recent Northern blot experiments from this laboratory have confirmed that the length of pMGA mRNA is consistent with a monocistronic RNA (M.D. Glew, P.F. Markham and I.D. Walker, unpublished data).

Perhaps most notable of all in respect of pMGA coding sequences 1.1–1.6 is the absence of internal stop codons which might have been expected had the pMGA system been functionally analogous to the pilin system of *N. gonorrhoeae*, the genome of which encodes multiple pilin gene variants, many of which contain one or more premature stop codons [6]. Clearly *M. gallisepticum* pMGA genes are under considerable selective pressure to maintain their ability to encode viable polypeptides rather than to simply act as an inert reservoir of sequence variability as is the case of *N. gonorrhoeae* pilin variants. The earlier observation from this laboratory of the expression of unique pMGA variants in 3 strains of *M. gallisepticum*, F, R and S6 [1], may thus be explained by switches in expression from one translationally functional pMGA gene to another rather than by a reciprocal exchange of DNA between one functional gene and other non-functional variants, as occurs between the pilin genes of *N. gonorrhoeae*. Experiments are now underway to investigate how the control of pMGA expression is accomplished in *M. gallisepticum* cells.

The Southern blot patterns of Fig. 2 do not comment on the details of such putative pMGA gene switching events. They do, however, demonstrate the fact that alterations of pMGA gene sequences probably occur commonly as *M. gallisepticum* cells respond to environmental challenge. It is tempting to invoke the host immune system as the primary means of selecting *M. gallisepticum* clones with an antigenically altered surface generated by pMGA gene switching events. However, no attempt was made in this work to compare the degree of polymorphism observed with pMGA-DNA probes to that observable with other probes which detect genes for proteins which can not act as targets for antibodies (e.g. which encode cytoplasmic proteins). In addition there has been no formal demonstration as yet that the immune status of host birds can bias the pMGA phenotypes of *M. gallisepticum* clones isolated from such animals, as compared to clones isolated from non-immune animals. It is attractive nevertheless to interpret the divergence between the pMGA gene clusters of *M. gallisepticum* isolates as indicating at least the potential of this organism to exploit a high level of pMGA gene alteration to facilitate adaptation to environmental stress. It should be noted that the stringency conditions used to obtain the Southern blots of Fig. 2 were low enough to permit imperfect base pairing between probes and target DNA: thus the detection of multiple pMGA- containing fragments was facilitated. In order to be sure that the hybridization stringency was nevertheless high enough to prevent irrelevant binding reactions, two probes corresponding to non-overlapping regions of the pMGA1.2/1.3 genes were used. That both probes produced very similar patterns when used to examine the same restriction digest is strong evidence that most or all of the fragments detected in this experiment are genuinely related in sequence to pMGA. The combined length of the pMGA restriction fragments from individual channels in Fig. 2 ranges from 100 kb to 120 kb. Assuming that the average length of a pMGA gene (including the intergenic region) is around 2.4 kb (as observed in the three genes for which we have the complete sequence) the number of pMGA genes within the *M. gallisepticum* genome can be estimated to be as many as 50. This is about 10% of the genome's total coding potential. Recent work from this laboratory has revealed that all pMGA genes are clustered in one or at most two separate segments rather than interspersed throughout the genome (G. F. Browning, P.F. Markham and K. G. Whithear, unpublished data).

4.2. The pMGA intergenic regions

The sequences of the pMGA intergenic regions elucidated in this work are aligned in Fig. 3 to maximise sequence homology. Considerable length variation is apparent between homologous regions. The presence of a variable number of GAA repeats is a prominent characteristic of the middle section of all of these intergenic regions but the significance of these repeats is unclear. The 200–250 nucleotides following the stop codon of the sequence preceding each intergenic region are relatively poorly conserved. A search for motifs within this region resembling *E. coli* rho-independent transcription stops revealed a number of palindromes in intergenic regions between pMGA1.6 and 1.2 and pMGA1.2 and 1.3 which might encode mRNA capable of forming base-paired terminators. Data is not yet available on this segment of the pMGA1.1 gene.

Within the last 3′ 100 or so nucleotides of the intergenic regions (Fig. 3) several possible transcriptional elements were identified. Firstly a −35 hexanucleotide consensus sequence CCGATT was found separated by 17 nucleotides from a −10 hexanucleotide TATATA consensus followed by about 50 nucleotides before the GUG translational start codons of all pMGA-like genes examined. The consensus *E. coli* −35 and −10 hexanucleotide sequences are, respectively, TTGACA and TATAAT, and their separation distances varies in *E. coli* between 16–18 nucleotides. The sequences of neither the putative −35 hexanucleotide nor the −10 hexanucleotides of the pMGA1.1 gene were unique. The sequences of the presumptive −10 and −35 boxes in Fig. 3 (boxed in dotted lines) are by no means a perfect match to known consensus *E. coli* promoter motifs and should be considered, at best, notional. Indeed upstream (5′) of these elements in Fig. 3 another possible −10, −35 pair of sequence motif exists (boxed in solid lines). Insufficient background data exists as yet in mycoplasma species to confidently predict which hypothetical set of promoter elements is most likely to be relevant to pMGA gene expression. Studies now in progress in this laboratory on pMGA mRNA size and properties may shed some light on which of the indicated promoter elements in Fig. 3 are relevant. For the sake of simplicity what follows assumes that the promoter elements of Fig. 3 (boxed in dotted lines) are correct.

A unique feature of the pMGA1.1 sequence relative to all other known pMGA-like sequences, which is located between the putative promoter region and the translational start codon, is the occurrence of an A nucleotide, 3′ distal to the −10 box of a proposed promoter region of the pMGA1.1 gene (that boxed in dotted lines). As the data of Fig. 3 indicates, other homologous sequences either contain a single base deletion at

this point (pMGA1.2) or contain G nucleotides (pMGA1.3-pMGA1.5). It is interesting that this position may be at or close to the point at which RNA polymerase begins transcription (5–8 nucleotides after the −10 box). The specificity properties of this enzyme in *M. gallisepticum* are not known but in other species there is a very strong bias toward the insertion of a purine at position 1 of mRNA molecules. Mammals strongly favour A whereas bacteria most commonly use A or G. If *M. gallisepticum* RNA polymerase were to have an unusual but obligate requirement for a T nucleotide located at the transcription initiation site on the DNA strand to be copied then this would account for the selective expression of the pMGA1.1 gene in the S6 strain. Whether this model to explain the selectivity of pMGA gene expression in *M. gallisepticum* is correct must await further detailed investigation in S6 and in other strains which express different variants of pMGA.

M. hyorhrinis also exhibits notable structural variation in surface lipoproteins (Vlp's) [7]. The Vlp system of *M. hyorhrinis* differs from the pMGA system of this paper in substantial respects but shares with *M. gallisepticum* the ability to switch on and off particular coding (Vlp) sequences. These switches occur frequently and involve additions or deletions within 5′ non-coding segment located between the -10 and -35 consensus promoter motifs on an A-rich segment. Indeed the deletion of a single A nucleotide from within this region was sufficient to extinguish the expression of a Vlp coding sequence. The mechanism of switching of Vlp genes in *M. hyorhrinis* seems to involve stochastic mutations occurring at high frequency at non-coding sites associated with transcriptional initiation. In this respect at least the mechanism of alteration of pMGA gene expression in *M. gallisepticum* may resemble the process in *M. hyorhrinis*.

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