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VARIABLE DNA SEQUENCE OF THE *pMGA* GENE IN *Mycoplasma gallisepticum*

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A B S T R A C T

Specific fragments of *Mycoplasma gallisepticum* genomic DNA, representing different regions of *pMGA1.1* and *pMGA1.2* genes, were amplified by Polymerase Chain Reaction (PCR). Using sequence analysis of *pMGA* terminal domains, differences in nucleotide sequences among strains of *M. gallisepticum* were identified. Restriction analysis revealed polymorphism at the 5′-end of the *pMGA* coding region. By means of sequence analysis it was established that the most variable part of *pMGA* sequence was the 5′-end of the *pMGA* gene, the region coding amino-terminal part of pMGA protein, while the 3′-end of the gene was conserved among various *M. gallisepticum* strains analysed. In some strains insertions or deletions were found that indicate the diversity of the *pMGA* gene family and might be related to the variable expression of the *pMGA* gene family. Changes in nucleotide sequences of the *pMGA* genes were also found within isogenic lineages. Genotypic variability is also confirmed by results of the study of the gene product expression.

Key words: microbiology / mycoplasma / *Mycoplasma gallisepticum* / molecular genetics / genes / pMGA / sequencing

VARIABILNA NUKLEOTIDNA ZAPOREDJA GENA *pMGA* PRI BAKTERIJI *Mycoplasma gallisepticum*

I Z V L E Č E K

S polimerazno verižno reakcijo (PCR) smo pomnožili specifične odseke genomske DNK bakterije *Mycoplasma gallisepticum*, komplementarne genoma *pMGA1.1* in *pMGA1.2* in s sekvenčno analizo terminalnih regij gena *pMGA* ugotavljali razlike v nukleotidnih zaporedjih med sevi *M. gallisepticum*. Restrikcijska analiza je pokazala polimorfizem na 5′-koncu kodogene regije *pMGA*. S sekvenčno analizo smo ugotovili, da je najbolj polimorfen del nukleotidnega zaporedja *pMGA* 5′-konec gena, to je regija, ki kodira amino-terminalni del proteina pMGA, medtem ko je 3′-konec gena ohranjen pri proučevanih sevih *M. gallisepticum*. Pri nekaterih sevih smo opazili insercije oziroma delecije, ki prispevajo k raznolikosti genov iz družine *pMGA* in utegnejo biti povezane s pojavom variabilne ekspresije te genske družine. Variabilna nukleotidna zaporedja genov *pMGA* smo našli tudi v izogenih populacijah. Genotipsko variabilnost je moč podpreti z rezultati študije ekspresije genskih produktov tudi na fenotipski ravni.

Ključne besede: mikrobiologija / mikoplazme / *Mycoplasma gallisepticum* / molekularna genetika / geni / pMGA / sekvenciranje

INTRODUCTION

Mycoplasma gallisepticum is an economically important pathogen in commercial poultry flocks causing chronic respiratory disease in chickens and turkeys. Invasion of the upper respiratory tract of infected birds is characterized by lesions, drop of productivity and increased mortality. The ability of the organism to adhere on the host epithelial cells is of crucial importance for its pathogenicity. The terminal structure located at one end of the *M. gallisepticum* cell is physically involved in the pathogenicity of the organism by ensuring the close contact between the mycoplasma and the host cell (Razin and Barile, 1985). The major *M. gallisepticum* adhesin pMGA is surface exposed, 67 kDa protein (Markham *et al*., 1992), responsible for attachment of mycoplasma to the epithelial cells of the respiratory tract. According to the role of the terminal structure, pMGA is predominantly clustered at its surface, much like the pMGA counterpart P1 in the human pathogen *Mycoplasma pneumoniae* (Markham *et al*., 1992), to enable mycoplasma to persist in the respiratory tract of infected birds for a long time (Levisohn and Kleven, 1981). Studies on the antigenic behaviour of the pMGA revealed considerable phase- and size-variation in *pMGA* gene expression (Benčina *et al*., 1994). The major adhesin of *M. gallisepticum* is coded by the members of the *pMGA* gene family (Markham *et al*., 1993). A different number of the *pMGA* gene family members is present in different strains of *M. gallisepticum,* in some strains even up to 50 members (Markham *et al*., 1994). Coding sequences have been grouped in 3-4 genomic clusters and in 9 different types (*pMGA1.1* - *pMGA1.9*) (Glew *et al.*, 1998). In *M. gallisepticum* strain S6 has been estimated that the *pMGA* gene family contains 33 members comprising a total of 7.7% of the 1.030 kb *M. galllisepticum* genome (Bassegio *et al*., 1995). The expression of the different members of the *pMGA* gene family can be regulated through the presence of the pMGA-specific antibodies (Markham *et al*., 1998). These results indicate that extensive variation in adhesin gene expression plays a pivotal role in the immune evasion strategy of *M. gallisepticum*.

In our study we analysed genomic variation among 11 *M. gallisepticum* strains within the coding region of the *pMGA 1.1* and *pMGA1.2* genes. Based on our previous results which documented variable expression of *pMGA* genes in different *M gallisepticum* strains we tried to prove the assumption that mutations and recombinations play an important role in variable expression of *pMGA* genes.

MATERIAL AND METHODS

M. gallisepticum strains

In this study we used our own *M. gallisepticum* isolates (ULB 921, ULB 931, G11 and PPT_2), and *M. gallisepticum* strains obtained form S.H. Kleven, University of Georgia, Athens ($S6_{208}$, A5969, R_{K781}, F), J.M.Bradbury, University of Liverpool, ($S6_{JMB}$), and L. Stipkovits, Hungarian Academy of Science, Budapest ($S6_B$, X-95). Genomic DNA from different *Mycoplasma gallisepticum* strains was isolated according to the method described previously (Dovč *et al.*, 1991).

Amplification of *pMGA 1.1* and *pMGA 1.2* specific genomic fragments

Based on the published DNA sequence of *pMGA1.2* gene from *M. gallisepticum* strain S6 (GenBank L28424), two pairs of oligonucleotides were designed. The first pair of chimeric primers PMGA1 (5′-GAGGAAACAGCTATGACTACACCAACACCTAACCCAACACC-3′) and PMGA2 (5′-GTAAAACGACGGCCAGTCATCGGACTTACTTTTTCTGCTGG-3′) contained the *pMGA* specific sequence tailed with complementary M13 universal and reverse

sequences. PMGA1 and PMGA2 were used for in vitro amplification of the 1200 bp long 5′-end of the *pMGA1.2* gene between the nucleotides 1426 and 2571. Due to the 92.3% sequence identity of the oligonucleotide PMGA1 to the *pMGA1.1* gene at position 262-287 and 100% sequence identity of the oligonucleotide PMGA2 to the *pMGA1.1* gene at position 1426 and 1450 we were able to co – amplify both genes in some *M. gallisepticum* strains. The 576 bp long fragment at the 3'-end of the *pMGA* gene including a part of the intergenic region was amplified using the primers PMGA3 (5′-ATGTGGGTGGAACTGGTGCTCG-3′) and PMGA4 (5′-AAAATTCCCAAAA TAAAAAAAACATC-3′). The annealing temperature for both pairs of primers was 54°C.

Restriction fragment analysis

RFLP analysis of the amplified region was performed using *Ava*I, *Dde*I, *Dra*I, *Hae*III, *Hha*I, *Hinc*I, *Hin*fI *and Taq*I. The most informative for the differentiation of *M.gallisepticum* strains was restriction with *Dde*I resulting in 4 to 6 fragments. Restriction fragments were separated either on 2% agarose or 6% polyacrylamide gels followed by silver or Ethidium bromide staining.

Sequence analysis of the *pMGA* terminal domaines

Sequence analysis was performed using standard dideoxynucleotide chain-termination method and DIG *Taq*DNA Sequencing Kit for non radioactive DNA cycle sequencing. Reactions were run on the PTC-100 thermal cycler (MJ Research) for 30 cycles with annealing temperature 58°C. Sequencing reaction products were separated on GATC 1500 Direct blotting apparatus. Sequence data were analysed using DNASIS™ software (Hitachi-LKB).

RESULTS AND DISCUSSION

The 1200 bp long genomic fragment representing the 5′-end of the *pMGA* gene from 11 *M. gallisepticum* strains was amplified *in vitro*. The selected primers PMGA1 and PMGA2 enabled preferentially amplification of the *pMGA 1.2* gene variant (nt 1386 - 2571) but in some strains we observed also co-amplification of more than one gene variant due to the high sequence homology between *pMGA 1.1* and *pMGA 1.2* in the primer region. Restriction fragment analysis of the 1200 bp long 5′-end region of the *pMGA* gene revealed *Dde*I polymorphism among the analysed *M. gallisepticum* strains. Three to five *Dde*I restriction sites were observed in this region.

DNA sequencing of the 5′-end region of the *pMGA* gene was performed for *M. gallisepticum* strains $S6_{208}$, $S6_B$, A5969, R_{K781} - low and high passage, X-95, ULB 931- *in vivo*, third and 13th passage, ULB 921 - *in vivo* and eight passage, G11 and PPT_2. The central part of the *pMGA* gene was sequenced in two strains (A5969 and R_{K781}). The nucleotide sequences were aligned to the *pMGA1.1* and *pMGA1.2* reference sequences from *M. gallisepticum* strain S6. The nucleotide sequence identity with the *pMGA1.2* gene was estimated to be 96% for the strains ULB 931-13th passage, ULB 921 - *in vivo* and eight passage and G11, 95% for the strain PPT_2, 94% for the strains $S6_B$ and A5969 and 69% for the low passage of the strain R_{K781}.

The nucleotide sequences from the strains A5969 and $S6_B$ represented a combination between the *pMGA1.1* and *pMGA1.2* sequences. Both strains showed considerable sequence differences in comparison to the reference sequence of *M. gallisepticum* strain S6 at the very 5′-end of the *pMGA1.2* gene. In the strain A5969 an insertion of 24-27 nucleotides with the nucleotide sequence for the last 20 nucleotides 5′-AACCAAAACCAGATCCAATG-3′ was found.

According to the *pMGA1.2* reference sequence the 3′-end of the insertion is at the nucleotide position 1415. Following the insertion the *pMGA1.1* specific nucleotide sequence 5′-CCAAACCCTCCTAG-3′ is proceeded by the *pMGA1.2* specific deletion of the triplet AAT. At least one more switch from the *pMGA1.2* sequence to the *pMGA1.1* sequence was identified further downstream. In the strain $S6_B$ the *pMGA1.2* specific T at position 1491 and *pMGA1.1* specific G at position 1530 were found, suggesting the recombination between both *pMGA* variants. In addition, 8 point mutations at positions 1550, 1582, 1587, 1591, 1596, 1622, 1695 and 1755 were detected. Due to the transition from G to A at position 1550 the first *Dde*I restriction site at position 1546 is abolished in strains $S6_B$ and A5969.

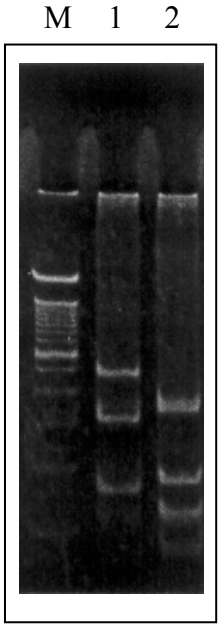

Figure 1. Restriction fragment analysis of the 5′-end of the *pMGA* gene after *Dde*I digestion. M = 100 bp ladder, 1 = low passage of the *M. gallisepticum* strain R_{K781}, 2 = *M. gallisepticum* strain $S6_{208}$.

Slika 1. Analiza restrikcijskih fragmentov 5′- konca gena *pMGA* po cepitvi z *Dde*I. M = velikostni marker 100 bp, 1= nizka pasaža seva R_{K781} *M. gallisepticum*, 2 = sev $S6_{208}$ *M. gallisepticum*.

We observed considerable nucleotide sequence differences between the low and high passage of the *M. gallisepticum* R strain. The region downstream from the nucleotide 1493 (5′-TCAAGAATTAGCTGCTGC-3′) is relatively conserved in both passages of the R strain. The low passage variant is coding for more Pro and Gly residues in this region compared to the high passage. The low passage of the *M. gallisepticum* R_{K781} strain is characterized by a 6 bp deletion between the nucleotide positions 2373 and 2382 in the *pMGA1*.1 reference sequence (GenBank Acc. No. L28423). In the R strain also two *Dde*I restriction sites at positions 1615 and 1666 are abolished.

In the 13th passage of the strain ULB 931 a G at position 1530 was replaced with an A causing a switch from *pMGA1.2* to *pMGA1.1* type. The nucleotide sequence shift in the low passage of this strain is a consequence of an 12-15 bp insertion in the 5′-region of the gene. The sequence in this region was difficult to read because of co-amplification of two genes.

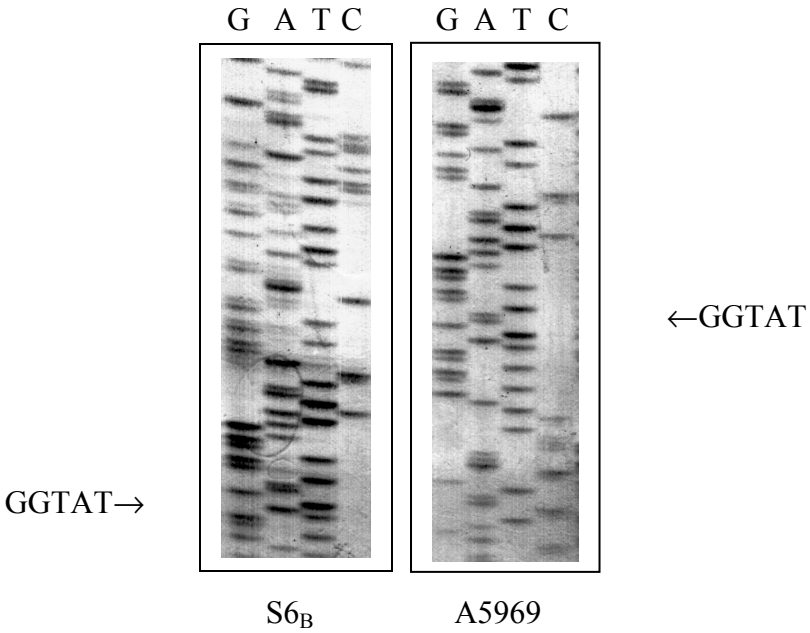

Figure 2. Shift of the GGTAT motif due to the insertion of 24 - 27 bp at the 5′-end of the *pMGA* gene in *M. gallisepticum* A5969 strain. Sequence of the strain $S6_B$ served as a control.
Slika 2. Premik motiva GGTAT (nt 1434) zaradi insercije 24 - 27 bp v 5′-koncu gena *pMGA* pri sevu A5969 *M. gallisepticum*. Za primerjavo smo uporabili sev $S6_B$.

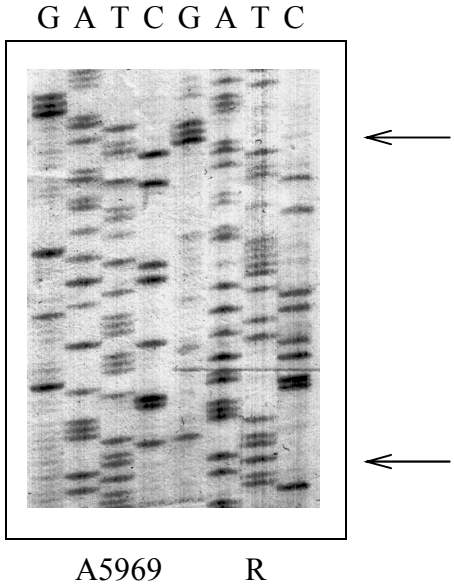

Figure 3. 6 bp deletion in the central region of the *pMGA* gene from the low passage of the *M. gallisepticum* R strain. Strain A5969 served as a control.
Slika 3. Delecija 6 bp v osrednjem območju *pMGA* nizke pasaže seva R *M. gallisepticum*. Za primerjavo smo uporabili sev A5969.

The 3′-end of the *pMGA* gene (nucleotides 2800 - 3376) coding the C-terminal part of the pMGA protein is conserved among *M. gallisepticum* strains. No sequence differences were found among the sequenced regions of four *M. gallisepticum* strains (A5969, $S6_{208}$, R_{K781}-low

passage, $S6_{JMB}$). Our results support the hypothesis that the 5′-region of the *pMGA* gene is the most variable region of the *pMGA* gene and to the great extent responsible for the genotypic variation among the *pMGA* genes in different *M. gallisepticum* strains.

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          2348      2358       2368       2378      2388
R(K781)  CCCTTAtAAGtTAGTTAAAAATAGTGAT--AgTG----TTGGTTT
         ||||||||||||||||||| | ||||||| | ||    | |||||
A5969    CCCTTAtAAGtTAGTTAATACTAGTGATCAAaTGAAACTAGGTTT
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Figure 4. Sequence alignment of the central region of the *pMGA* gene bearing a 6 bp deletion in the *M. gallisepticum* R strain. Small letters represent DNA sequence differences between *pMGA1.1* and *pMGA1.2*.

Slika 4. Primerjava nukleotidnih zaporedij srednjega dela gena *pMGA*, ki ima pri sevu R delecijo 6 bp. Male črke predstavljajo razlike v nukleotidnem zaporedju med genoma *pMGA1.1* in *pMGA1.2*.

SUMMARY

Chronic respiratory infections in poultry caused by *Mycoplasma gallisepticum* indicate that this mycoplasma species can adapt rapidly to the changes occurring in the host organism. Recent studies have shown that *M. gallisepticum* has the ability to change its surface exposed, immunodominant proteins which are involved in attachment of mycoplasma to the tracheal epithelium of the host. Antigenic variation of the membrane proteins plays a central role in immune evasion strategy of *M. gallisepticum.*

Hemagglutinin pMGA is one of the major membrane associated, variably expressed proteins of *M. gallisepticum,* important for the generating the disease of the host organism. Previous studies have found the large number of *pMGA* gene family members in the genome of *M. gallisepticum* which are coding for antigenic related variants of the immunodominant lipoprotein pMGA. In addition to the phase variable expression of the *pMGA* gene family members there is also the evidence that some new pMGA variants are generated by recombination between the members of the gene family.

In this study we tried to elucidate molecular background of the antigenic variation in *M. gallisepticum*. Using the polymerase chain reaction the genomic DNA representing parts of the *pMGA1.1* and *pMGA1.2* genes was amplified and DNA sequencing or the terminal parts of these fragments revealed nucleotide sequence differences among the *M. gallisepticum* strains. Restriction analysis of the 5′-end of the *pMGA* coding region revealed polymorphic *Dde*I restriction sites among different *M. gallisepticum* strains. The nucleotide sequence analysis confirmed restriction polymorphisms and demonstrated that the 5′-end coding the N-terminal part of the protein is the most diverse part of the *pMGA* gene. Among the *M. gallisepticum* strains included in this study we did not found any nucleotide sequence differences within the 3′-region of the gene. We conclude, therefore, that the C-terminal part of the pMGA protein is highly conserved in *M. gallisepticum*. DNA sequence analysis revealed nucleotide sequence differences in the *pMGA* coding region also within the isogenic populations. DNA sequence differences among different cultures of the same strain, caused by insertions and deletions demonstrate *pMGA* gene structure differences among the subpopulations. The genotype variation can also be confirmed at the protein level.

The importance of the variable expression of the *pMGA* gene family members is best illustrated by the fact that in the *M. gallisepticum* genome, which is close to the minimal required

genome size for the free living cell, up to 15% of the genome is occupied with the coding regions for the single surface exposed protein.

POVZETEK

Kronična narava okužb dihal perutnine, ki jih povzroča *M. gallisepticum*, kaže, da je ta vrsta mikoplazem sposobna hitrega odziva in prilagajanja na spremembe v gostitelju. Raziskave zadnjih let so pokazale, da ima *M. gallisepticum* izjemno sposobnost spreminjanja površinskih imunodominantnih proteinov, povezanih s pritrjevanjem te mikoplazme na trahealni epitel gostitelja. Antigenska variacija membranskih proteinov je eden od mehanizmov, s katerimi se *M. gallisepticum* izogiba imunskemu odzivu gostitelja.

Med najmočneje izražene proteine membranske frakcije tega mikroorganizma sodi hemaglutinin pMGA, ki se izraža variabilno in na ta način verjetno odločilno vpliva na odnos z gostiteljem. Dosedanje študije so pokazale, da genom *M. gallisepticum* vsebuje veliko število variant genov *pMGA*, ki kodirajo antigensko sorodne različice imunodominantnega lipoproteina pMGA. Poleg fazno variabilnega izražanja genov *pMGA* številne različice proteina pMGA nastajajo tudi z rekombinacijo med temi geni.

V pričujoči raziskavi smo proučevali genetsko ozadje antigenske variabilnosti *M. gallisepticum*. S polimerazno verižno reakcijo (PCR) smo pomnožili specifične odseke genomske DNK *M. gallisepticum*, komplementarne genoma *pMGA1.1* in *pMGA1.2*, in s sekvenčno analizo terminalnih regij gena *pMGA* ugotavljali razlike v zaporedjih nukleotidov med sevi *M. gallisepticum*. Restrikcija produktov PCR z encimom *Dde*I je pokazala polimorfizem na 5′-koncu kodogene regije *pMGA*. S sekvenčno analizo smo potrdili rezultate restrikcijske analize in ugotovili, da je najbolj variabilen del zaporedja nukleotidov *pMGA* 5′-konec gena, to je regija, ki kodira N-terminalni del proteina pMGA. Med proučevanimi sevi nismo našli razlik v zaporedju nukleotidov na 3′-koncu gena, to je v regiji, ki kodira C-terminalni del proteina pMGA, in v kratki robni intergenski regiji. Sklepamo, da je ta regija pri sevih *M. gallisepticum* manj variabilna, kot je N-terminalna regija. Z analizo zaporedja nukleotidov smo ugotovili razlike v kodogeni regiji *pMGA* tudi v izogenih populacijah. Različna zaporedja nukleotidov pri različnih kulturah istih sevov, ki so posledica insercij oziroma delecij delov genov *pMGA*, kažejo na spremembe v strukturi genov *pMGA* pri subpopulacijah. Genotipsko variabilnost je moč podpreti z rezultati študije ekspresije genskih produktov tudi na fenotipski ravni.

Dejstvo, da mikroorganizem s tako omejeno zmogljivostjo genetskega kodiranja pri nekaterih sevih nameni skoraj šestino genomske DNA za izražanje različic ene vrste proteina, kaže na izjemen pomen spremenljivega izražanja površinskih proteinov za preživetje *M. gallisepticum*.

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