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Mycoplasma synoviae HAEMAGGLUTINATION INVOLVES IMMUNODOMINANT SURFACE MEMBRANE PROTEINS UNDERGOING PHASE- SIZE- AND ANTIGENIC-VARIATION

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ABSTRACT

In the majority of *Mycoplasma synoviae (MS)* strains their VLHA haemagglutinins cleave into N-terminal and C-terminal parts. In MS strain ULB 925 (clone KF9) it was found that monoclonal antibodies (mAbs) 3E10, 5G6 and 4G2 recognise the C-terminal parts of its haemagglutinin, designated pMSA, as integral membrane proteins of 51 and 53 kDa. In the same MS clone mAb 125 recognized the N- terminal parts, designated pMSB, as integral membrane proteins of 47 and 49 kDa. However, in MS strain K1723 mAbs 3E10, 5G6 and 125 reacted with an uncleaved VLHA haemagglutinin of about 90 kDa. In MS strain ULB 925 clones their haemagglutinin- positive (HA^+) and haemadsorption- positive (HAD^+) phenotypes synthesised pMSA defined by mAbs. About 1 % of the populations which ceased to produce pMSA and produced truncated $pMSB_1$ forms (of 27 to 30 kDa) lost the HA^+ and HAD^+ phenotype. The switching of the synthesis of pMSA proteins was heritable and usually reversible. In strain ULB 925 (clone KF9) certain populations which ceased to produce pMSA defined by mAb 3E10 started to produce pMSB antigenic variant, recognised by another mAb. In MS strains, besides antigenic- variation of pMSA and pMSB, their size- variants were also observed and they were recognised by antibodies of chickens infected with MS.

Key words: microbiology / *Mycoplasma synoviae* / immunology / haemagglutination / membrane proteins / protein variation

HEMAGLUTINACIJA *Mycoplasme synoviae* JE POVEZANA Z IMUNODOMINANTNIMI POVRŠINSKIMI MEMBRANSKIMI BELJAKOVINAMI, KI SO FAZNO, VELIKOSTNO IN ANTIGENSKO SPREMENLJIVE

IZVLEČEK

Pri večini sevov *Mycoplasme synoviae* (MS) se njihovi VLHA hemaglutinini cepijo na N-terminalni in C-terminalni konec. Pri sevu ULB 925 (klon KF9) so C-terminalni deli, imenovani pMSA, integralne membranske beljakovine z molekulsko maso 51 in 53 kDa. Te beljakovine so prepoznavala monoklonska protitelesa (mAb) 3E10, 5G6 in 4G2. N-terminalni deli, imenovani $pMSB_1$, hemaglutinina istega klona so prav tako integralne membranske beljakovine z masami 47 in 49 kDa, ki jih prepoznava mAb 125. Pri sevu K1723 pa mAb 3E10, 5G6 in 125 reagirajo z necepljenim VLHA hemaglutininom z maso okrog 90 kDa. Pri sevu ULB 925 hemaglutinin pozitivni (HA^+) fenotip klonov in hemadsorbcijsko pozitivni (HAD^+) fenotip klonov sintetizirajo celoten (51–53 kDa) hemaglutinin pMSA. Približno 1 % populacije, ki pa ne proizvaja pMSA, proizvaja pa spremenjeno obliko $pMSB_1$ (z maso 27–30 kDa), izgubi HA^+ in HAD^+ fenotip. Prenehanje sinteze beljakovine pMSA je dedna in običajno reverzibilna lastnost.

Določene populacije seva ULB 925 (klon KF9), ki so prenehale sintetizirati beljakovino pMSA in jo mAb 3E10 prepoznavajo, so začele sintetizirati antigensko varianto beljakovine pMSB, ki jo prepoznavanjo druga mAb. Polega antigenske spremenljivosti beljakovin pMSA on pMSB smo pri sevih MS opazili tudi spremenljivost velikosti beljakovin. Različne variante teh beljakovin so prepoznavala tudi protitelesa piščancev, okuženih z MS.

Ključne besede: mikrobiologija / mikoplazme / *Mycoplasma synoviae* / imunologija / hemaglutinacija / membranske beljakovine / spremenljivost beljakovin

INTRODUCTION

Mycoplasma synoviae (MS) is a major pathogen of chickens and turkeys, causing significant economic losses to the poultry industry. Infection most frequently occurs as a subclinical upper respiratory infection, and vertical transmission of MS plays a major role in its spread in poultry flocks (Kleven, 1997). Natural MS infection has also been found in guinea fowl, ducks, geese, pigeons, Japanese quail and red-legged partridge with evidence of its vertical transmission in water-fowl (Pascucci *et al.,* 1976; Recce *et al.,* 1986; Benčina *et al.,* 1987; Benčina *et al.,* 1988a; Benčina *et al.,* 1988b; Poveda *et al.,* 1990). In chickens and turkeys, infection may become systemic and results in infectious synovitis involving primarily the synovial membranes of joints and tendon sheaths. Typically, MS colonises the epithelial linings of the respiratory tract, but may travel via the vascular system to a variety of organs and cause myocarditis, glomerulonephritis, extreme atrophy of primary lymphoid organs and meningeal or even systemic vasculitis (Kawakubo *et al.,* 1980; Kawakubo *et al.,* 1981; Kleven, 1997).

Chickens infected with MS responded with antibodies to more than 10 proteins, and clusters of integral membrane proteins of 41–52 kDa were shown to be the major immunogens (Avakian and Kleven, 1990a; Avakian and Kleven, 1990b; Avakian *et al.,* 1992; Gurevich *et al.,* 1995). On the other hand, comparison of MS isolates by immunoblotting indicated that several highly immunogenic integral membrane proteins vary in size and quantity (Avakian *et al.,* 1992; Noormohammadi *et al.,* 1997).

Like four other pathogenic avian *Mycoplasma* species, MS is also capable of haemagglutinating chicken and turkey erythrocytes, though this property varies, depending on the MS isolate (Rhoades, 1985). It has been reported that MS type strain WVU-1853 has two distinct groups of phase-variable major membrane proteins, of which the 50 kDa protein termed MSPA was proposed as a putative haemagglutinin (Noormohammadi *et al.,* 1997). A recent study has identified the *vlhA* gene which in MS strain WVU-1853 encodes haemagglutinin. It has also been proposed that posttranslational cleavage of the *vlhA* gene products generates MSPB (the 45–47 kDa N-terminal part) and MSPA (the 50 kDa C-terminal part) proteins (Noormohammadi *et al.,*1998). In MS strain ULB 925 we identified the cleavage site of haemagglutinin by direct amino acid sequencing of the N-terminal parts of the pMSA proteins (Benčina *et al.,* 1999). These proteins are homologues of MSPA proteins of MS strain WVU-1853 and are associated with the haemagglutination positive (HA^+) and haemadherence positive (HAD^+) phenotypes of MS strain ULB 925 clones (Narat *et al.,* 1998; Benčina *et al.,* 1999). We also reported that the HA^+ phenotype of MS strain AAY-4 induced experimental infectious synovitis in the chicken more frequently than did the HA negative (HA^-) phenotype (Narat *et al.,* 1998). Thus, it seems that the expression of proteins associated with the HA^+ phenotype might be associated with the pathogenicity of MS. Moreover, we reported that the N-terminal part of the haemagglutinin (homologue of MSPB) was a target of an early local antibody response in hock joints inoculated with MS (Narat *et al.,* 1998).

The present study was undertaken to investigate further the proteins associated with MS HA^+ and HAD^+ phenotypes and to estimate the frequency of their phase-variable expression. We also

investigated antigenic variants of haemagglutinins, as well as their size-variation in different MS strains.

MATERIAL AND METHODS

Mycoplasma strains

The *M. synoviae* strains F10-2AS, K2445, K2581, K2426, K2559 that were used, have previously been compared by immunoblotting in a study by Avakian *et al.* (1992). HA^+ and HA^- cultures of MS type strain WVU-1853 were received from Dr.J.M. Bradbury. MS strain K1723 which synthesises an uncleaved haemagglutinin (Noormohammadi *et al.,* 1997) was obtained at 8th passage from Dr.P.F. Markham. However, this strain originates from the trachea of a cock of heavy breed from Arkansas, USA and was isolated in 1982. Strain FMT is a reference MS strain used in several studies (Morrow *et al.,* 1990; Noormohammadi *et al.,* 1997). MS strains AAY-4 and ULB 925 (a reisolate of AAY-4 from the hock joint) have been used previously and also described (Narat *et al.,* 1998; Benčina *et al.,* 1999). Other MS strains isolated from different breeds of naturally infected chickens in Slovenia were JAHO-1 and ULB 932 (hock joints), ULB 933, ULB 9122 and PAA-2 (trachea), AAY-30 (yolk sac) and MIE 61 (egg yolk). Strains AAY-4, AAY-30 and ULB 9122 had identical DNA banding patterns with the arbitrarily primed polymerase chain reaction (Fan *et al.,* 1995). Frey's medium containing 15 % swine serum was used in agar and broth forms for isolation and cultivation (Frey *et al.,* 1968).

Table 1. *M. synoviae* proteins recognised by mAbs

Monoclonal antibody		Proteins recognised by mAbs					
Designation	Isotype	Designation[a]	Strain ULB 925 clones			Strain K1723	Strain WVU-1853
			KF 9 (HA^+)	KF 6 (HA^-)	KF 9/4 (HA^-)	Clone K23 (HA^+)	Clone JMB-1 (HA^+)
3E10	IgG_1	$pMSA_{1(a,b)}$	51, 53[b]	NP[c]	NP	90	50
5G6	IgG_{2a}	pMSA	51, 53	NP	NP	90	50
4G2	IgG_1	pMSA	51	NP	NP	NP	50
3B4	IgM	pMSB	NP	NP	50	90	50
4B3	IgG_1	?	67, 85	67, 85	67, 85	67, 85	67, 85
125[f]	IgG_{2b}	$pMSB_{1(a,b)}$	47, 49	27, 30	27, 30, 47	90	45, 47
5[g]	IgG_{2a}	?	48	48	48	48	47
3[g]	$IgG_{1(2a)}$	?pMSB	50	NP	NP	ND[d]	47
10[g]	IgG_1	?	48	48	48	ND	47
50[f]	$IgG_{2(a,b)}$	MSPB[h]	NP	NP	48	NP	45, 47
5F7[g]	IgG_{2a}	?pMSB	NP	NP	NP	NP	45
R(CCM-2)	IgG_1	EF-Tu[e]	43	43	43	43	43

[a] = Only proteins of KF 9 clone with determined or deduced amino acid sequences are labelled with distinct designations. Questionmark (?) indicates uncertain classification. The N-terminal amino acid sequences: $pMSA_{1a}$ – SENETXXA; $pMSA_{1b}$ – SPAEK; $pMSB_2$ – MGDQTPAPA; EF-Tu –AKLDFDRSKEHVNVGTIGHV (see references Noormohammadi *et al.,* 1997; Narat *et al.,* 1998; Benčina *et al.,* 1999).

[b] = Molecular masses of proteins in kilo Daltons recognised in immunoblotting. [c] = no protein recognised.

[d] = not determined. [e] = The N-terminal amino acid sequence has been determined (Benčina *et al.,* 1999).

[f] = Received from D.H.Ley (Gurewich *et al.,* 1995; Noormohammadi *et al.,* 1997).

[g] = Received from Gy. Czifra (Czifra *et al.,* 1994). [h] = MSPB is a homologue of pMSB.

Other *Mycoplasma* species

Species used in this study were: *M. gallisepticum* S6 (culture 208, Benčina *et al.,* 1994b); *M. iowae* (I-695), *M. pneumoniae* (type strain FH), *M. pulmonis* (PG 34) and *M. hyopneumoniae* (strain J).

Preparation of polyclonal and monoclonal antibodies

Production of rabbit antibodies to MS strains WVU-1853 and AAY-4 has been described previously (Benčina *et al.,* 1987). Chicken antisera to MS strains WVU-1853, AAY-4 and F10-2AS were raised in 2–3 week-old mycoplasma-free broiler chickens by inoculating birds with 10^7 to 10^8 colony forming units (CFU) of the appropriate MS strain into the hock joints (Narat *et al.,* 1998). Serum or synovial fluid samples were collected weekly and tested using different serological assays.

Monoclonal antibodies (mAbs) 3E10, 5G6, 4G2 and 4B3 were raised against haemagglutinating proteins of about 67 kDa of MS strain AAY-4 (Benčina *et al.,* 1994a) using an already described procedure (Benčina *et al.,* 1994b). MAb 3B4 was obtained following immunization of mice with about 10^9 heat-inactivated cells of MS strain AAY-4. Hybridomas were screened for antibody production to particular MS antigens by the dot immunobinding assay (DIBA), indirect immunoperoxidase assay (IIPA) with intact MS colonies, and by immunoblotting of proteins as has been described earlier (Benčina *et al.,* 1994b). MAbs produced in this laboratory, those received from Dr. Gy. Czifra and Dr. D.H. Ley (Czifra *et al.,* 1994; Gurevich *et al.,* 1995) are described in Table 1. MAb designated mAb R, which reacts with the cytoplasmic EF-Tu protein invariably expressed in all tested MS strains (Benčina *et al.,* 1999), was purchased (Ridascreen kit, Biopharm).

SDS-PAGE and immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed using the PhastSystem (Pharmacia, LKB) as described elsewhere (Benčina *et al.,* 1994b; Narat *et al.,* 1998). In consecutive immunoblotting studies (two colour staining) the first chromogene substrate was 3,3'-diaminobenzidine (DAB, Sigma). The membrane was then washed 3 times in phosphate buffered saline (PBS, pH 7.4) with 0.05 % Tweeen 20, after which the second immunostaining was conducted. This included another mAb (different from that used in the first reaction), an appropriate antimouse Ig (IgG or IgM) horseradish peroxidase (HRP) conjugate (Sigma). For the second staining peroxidase substrate TrueBlue™ (Kirkegaard & Perry laboratories) which gave a blue colour was used and was easily distinguishable from the brown signals developed in the first reaction (Benčina *et al.,* 1999).

Indirect immunoperoxidase assays

IIPA was used for screening colonies of MS strains for expression of proteins bearing surface epitopes recognized by mAbs (Benčina and Bradbury, 1991; Benčina *et al.,* 1994b). For two-colour staining the first reaction was developed using DAB, while the second was conducted using a different mAb, an appropriate HRP-conjugate and TrueBlue substrate. This enabled a clear distinction to be made between colonies expressing surface proteins defined by different mAbs.

Haemagglutination and haemadsorption

HA assay was performed using a 0.5 % suspension of chicken erythrocytes in plastic microplates (V bottom) at room temperature using 50 µl volumes of serially diluted MS

antigens. MS cells were washed 3 times in PBS pH 7.4 and assayed as pelleted or 10 times or 100 times concentrated broth cultures (Rhoades, 1985). HAD was also conducted at room temperature by a procedure described elsewhere (Gardella and DelGuidice, 1983), or by modified procedure in which agar blocks with MS colonies were used. The blocks bearing well separated MS colonies were prepared as for IIPA (Benčina and Bradbury, 1991). Then the blocks were overlayed with a 0.5 % suspension of erythrocytes for 30 minutes. Following careful washing of the blocks with PBS, haemadsorption was graded from 1+ to 3+ as recommended (Gardella and DelGuidice, 1983).

Haemagglutination- inhibition and inhibition of haemadsorption

HA-inhibition (HI) was performed as a micromethod using 4 HA units. The HI titers of mAbs, chicken serum or rabbit antisera were determined using different HA antigens as used for HA above. Inhibition of HAD (IHAD) was conducted using colonies of different MS strains and their clones which were HAD^+ as determined by the HAD assay above. To determine whether mAbs or antisera contained HAD inhibiting antibodies, MS colonies were incubated with them (in different dilutions, e.g. mAbs supernatants from undiluted to up to 1:128 dilution) for 30 minutes. The colonies were then incubated with 0.5 % suspension of chickens erythrocytes. As positive controls MS colonies incubated with PBS instead of antibodies were used. HAD and IHAD were estimated as recommended (Gardella and DelGuidice, 1983).

Triton x-114 phase partitioning

Integral membrane proteins from MS strains were extracted using Triton x-114 (Tx-114, Sigma) as previously described (Avakian *et al.,* 1992), though the detergent phases were washed 3 times. Proteins were extracted from the Tx-114 phase using methanol/chlorophorm and the procedure described elsewhere (Wessel and Fluegge, 1984).

Cloning of *M. synoviae* strain

MS strains were cloned to obtain HAD^+ and HA^+ phenotypes, as well as clones expressing surface proteins as defined by mAbs using IIPA and immunoblotting (Benčina *et al.,* 1994b; Narat *et al.,* 1998). For MS strain ULB 925 at least 8 different clones of each generation were assayed for HAD and HA phenotypes. In addition, expression of pMSB and pMSA proteins defined by mAbs (Benčina *et al.,* 1999) was analysed for hundreds of clones of the ULB 925 strain. Thus, phase-variation of pMSA proteins was studied in the progeny lineages derived from the parental clone KF for 10 consecutive generations. MS strain K1723 (synthesising an uncleaved haemagglutinin) was filter-cloned (0.2 μm pore size filters, Minisart, Sartorius) to obtain 48 clones, but only one clone (K23) reacted with mAb 3E10. Following the next filter-cloning of clone K23, tens of its subclones were examined for HA, HAD and expression of the pMSA recognised by mAb 3E10 in IIPA and immunoblotting.

RESULTS

pMSA proteins recognised by mAbs

MS strain ULB 925 clone KF9 (HA^+ culture whose HA titer of pelleted cells was 4×10^4) was used to determine pMSA proteins recognised by mAbs. In this MS culture the N-terminal amino acid sequences were determined for 3 pMSA forms (Benčina *et al.,* 1999). In the immunoblotting mAb 3E10 reacted with two proteins designated $pMSA_1$ which had molecular weight of 51 and 53 kDa. Because they had different N-terminal sequences they were designated

$pMSA_{1a}$ and $pMSA_{1b}$ (Table 1). On the other hand, in MS strain K1723, which synthesises an uncleaved haemagglutinin (VLHA), mAb 3E10 reacted with a much longer protein than pMSA of ULB 925 strain. Indeed, in the former strain clone K23 (HA^+ culture with HA titer 2 x 10^4) mAb 3E10 reacted with a protein of about 90 kDa (Fig. 1A). In the same MS clone mAb 125 (to pMSB proteins) also reacted with a protein of about 90 kDa. Thus, MS strain K1723 produced a 90 kDa VLHA haemagglutinin which did not cleave into pMSB and pMSA. MAb 5G6 in MS clones KF 9 and K23 reacted with proteins which could not be distinguished from those recognised by mAb 3E10 (Fig. 1B). However, mAbs 3E10 and 5G6 did not react with the same epitope, and in certain clones of AAY-4 strain the proteins recognised by mAb 3E10 differed in molecular weight from those recognised by mAb 5G6 (data not shown). In clone KF 9 mAb 4G2 reacted with a protein which comigrated with $pMSA_{1a}$ recognised by mAb 3E10, but it did not react with $pMSA_{1b}$. In immunoblotting mAb 4G2 did not react with proteins of MS strain K1723. In addition to $pMSA_1$ proteins, clone KF 9 also synthesised a third distinct pMSA protein designated $pMSA_2$. It had the N-terminal sequence SENKLIXXA (X indicates amino acids which could not be determined). Thus, clone KF 9 synthesised at least three different pMSA proteins with distinct amino acid sequences encoded by different *vlhA* sequences.

pMSB proteins recognised by mAbs

In MS strain ULB 925 clone KF 9 its pMSB proteins are longer than the EF-Tu protein but shorter than pMSA proteins (Benčina *et al.,* 1999). In this clone mAb 125 reacted with two proteins with molecular weight of about 47 and 49 kDa (Fig. 1B). These two proteins were designated $pMSB_{1a}$ and $pMSB_{1b}$, respectively. Their N-terminal amino acid sequences were blocked. Their N-terminal amino acid sequence (predicted from the sequence of the *vlhA* gene in KF 9 clone) would be C_1GDQTPAPA (Benčina *et al*., 1999). For the third protein, designated $pMSB_2$, a very similar N-terminal sequence was determined by microsequencing i.e. M_1GDQTPAPA. This protein could be encoded by the *vlhA* gene only if its codon for cysteine (TGT) would change into the codon for methionine (ATG). MAb 50, which in MS type strain WVU-1853 reacted with MSPB (homologues of pMSB) in immunoblotting, recognised no protein in clone KF 9 or in MS K1723 clone K23.

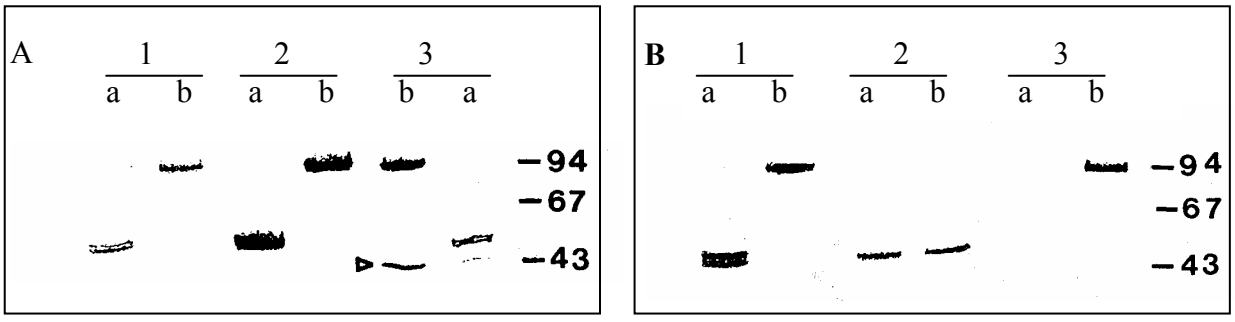

Figure 1. Reactions of proteins of *M. synoviae* strains ULB 925 and K1723 with monoclonal antibodies in immunoblotting. Lanes a, strain ULB 925 (HA^+ clone KF 9); lanes b, strain K1723 (HA^+ clone K23). **Panel A**: 1, reaction with mAb 5G6; 2, reaction with mAb 3E10; 3, reactions with mAbs 3E10 and then with mAb R. Note: in lane 3a the antigen was used more diluted (1:5) than in the other a lanes. The arrowhead indicates EF-Tu protein. **Panel B**: 1, reaction with mAb 125; 2, reaction with mAb 5; 3, reaction with mAb 3B4. Molecular masses (in kiloDaltons) are indicated at the right. Note: in MS strain K1723 (lanes b) a protein of about 90 kDa reacted with mAbs which recognised $pMSA_1$ and $pMSB_1$.

Other *M. synoviae* proteins recognised by mAbs

MAb 4B3 in MS strains ULB 925 (clone KF 9) and K1723 (clone K23) reacted with proteins of about 67 kDa and 85 kDa. In clone KF 9 a minor reaction also occurred with a protein of about 53 kDa. MAb 3B4 in MS strain K1723 reacted with a protein of about 90 kDa, but in immunoblotting recognised no protein in the KF 9 clone (Fig. 1B). However, in certain subclones of KF 9 clone which did not produce $pMSA_1$ (detected by mAb 3E10) mAb 3B4 reacted with a protein of about 50 kDa.Tx-114 partitioning showed that they were integral membrane proteins. Our other observations suggested that mAb 3B4 recognised pMSB antigenic variants which are usually not associated with the HA^+ phenotype (see Antigenic variants). MAb 3 in clone KF 9 reacted with an integral membrane protein of about 48 kDa (Fig. 3C). This variably expressed protein might belong to the pMSB proteins, but was not assigned because its amino acid sequences are not known. MAb 5F7 in immunoblotting did not react with KF 9 clone or K1723 strain (several clones) proteins. MAbs 5 and 10 recognised 45 kDa proteins invariantly expressed in all MS strains (Fig. 1B). These proteins are immunogenic for chickens, but their encoding genes remain to be identified and characterized.

Phase-variation in the expression of pMSA and pMSB proteins

Several MS strains were cloned and screened with IIPA using mAbs to pMSA and pMSB proteins to determine the surface expression of these proteins. The most extended analyses were performed in isogenic lineages of MS ULB 925 strain clone KF. This parental clone originated from a single colony and had a high HA titer (pelleted cells had an HA titer of $4x10^4$). In addition, about 99 % of the colonies of KF clone had the HAD^+ phenotype. In IIPA about 99 % of the colonies reacted with mAbs 3E10, 5G6 and 4G2, indicating that the relevant pMSA proteins were surface exposed and synthesised by the great majority of the population. Subcloning of the HAD^+ colonies of the parental clone established 11 subclones, including clone KF 9, in which 95–99 % of the colonies were HAD^+ and reacted with mAbs 3E10, 5G6 and 4G2 (Fig. 2A and B). From one HAD^- colony of the parental clone KF, a subclone designated KF 6 was obtained. Only about 1 % of the colonies of KF 6 clone was HAD^+ and a similar percentage of the colonies immunostained with mAbs 3E10, 5G6 and 4G2. In contrast, mAb 125 (recognising $pMSB_1$ proteins) immunostained rather uniformly all colonies of clone KF 6, as well as those of clone KF 9. Further analyses of subpopulations deriving from clones KF 9 or KF 6 revealed that the HAD phenotype and the expression of pMSA proteins detected by mAbs behaved as a heritable property. Thus, subclones of a single colony expressing pMSA with an epitope defined by mAbs expressed such pMSA forms in 95 to 99 % of colonies in the next generation. The minority of the population (1 to 5 %) revealed switching of the appropriate pMSA expressed at the surface of the colonies, from positive expression to negative, or the reverse. Moreover, in the great majority of the colonies from any subclone of KF 9 clone, a positive correlation between immunostaining with mAb 3E10 and HAD^+ phenotype was observed (Fig. 2C). Interestingly, in certain KF 9 clone subclones those colonies which did not react with mAb 3E10 and had HA^- phenotype eventually reacted with mAbs 50 and 3B4. The alternative expression of either pMSA detected by mAb 3E10 or of pMSB detected by mAb 50 was demonstrated by consecutive IIPA using mAbs one after another. Thus, colonies which did not react with mAb 3E10, then reacted with mAb 50. However, rare colonies did not stain with either of the two mAbs (not shown). Proteins recognised by mAbs 3E10, 5G6 and 4G2 were usually coexpressed and associated with the HAD^+ phenotype in MS strains AAY-4, ULB 925 and K1723. In several other MS strains, including WVU-1853, the positive reaction of colonies with mAb 3E10 was not always positively correlated with their HAD^+ phenotype. In addition, mAbs 125 and 50 in IIPA did not show reactions relevant to the HAD^+ phenotype of an

appropriate MS strain. MAb 3B4 reactions in IIPA indicated phase-variable expression of relevant proteins in all MS strains examined so far. However, in most ULB 925 strain clones mAb 3B4 reacted predominantly with colonies with the HAD^- phenotype. In rare MS cultures proteins recognised by mAbs 3B4 and 50 were coexpressed. Such coexpression was never observed for clones of the MS K1723 strain. The initial culture of this strain derived from the eighth passage contained less than 1 % colonies which reacted with mAbs 3E10 and 5G6. Following filter cloning 48 clones were established but only one clone (K23) which reacted well with mAbs was obtained. Further examinations revealed that the expression of proteins (about 90 kDa) defined by mAbs 3E10 and 5G6 is heritable, since up to 99 % of colonies reacted with these mAbs. The presence of the 90 kDa protein recognised by these mAbs is associated with the HAD^+ and HA^+ phenotypes.

Figure 2. Indirect immunoperoxidase staining of *M. synoviae* strain ULB 925 colonies with monoclonal antibodies. **Panel A**, in clone KF 9 mAb 3E10 immunostained the majority of colonies except the colony marked with an arrowhead. **Panel B**, mAb 5G6 in the same culture also reacted with the majority of colonies (the arrowhead indicates the unstained colony). **Panel C**, reactions of mAb 3E10 and haemadsorption were positively correlated in ULB 925 strain cultures. Note: on stained colonies (dark colonies) attached erythrocytes can also be seen as bright round dots. Unstained colonies (some are indicated by arrowheads) did not bind erythrocytes.

Proteins associated with haemagglutination

From the parental clone KF (strain ULB 925) with the HA^+ phenotype (HA titer 4 x 10^4) 11 HA^+ colonies and one HA^- clone were established. The HA^- clone (designated) KF 6 had an HA titer of less than 10^2 (for pelleted cells). In contrast clone KF 9 and other HA^+ clones had HA titer of the parental clone KF. Thus, isogenic clones KF 9 (with well defined pMSA and pMSB proteins) and KF 6 (with a some 1000 times lower HA titer) were selected to identify proteins associated with the HA^+ phenotype. Cells of these two clones were subjected to Tx-114 partitioning to obtain their integral membrane proteins. Examination of these proteins by SDS-PAGE and by Coomassie staining revealed a striking difference (Fig. 3A). The HA^+ clone (KF 9) had abundant proteins of 47–49 and 51–53 kDa which were much less expressed in HA^- clones (KF 6). This was particulaly evident for the 51–53 kDa proteins which were lacking in clone KF 6. On the other hand, there was no difference in the synthesis of membrane proteins of about 85 kDa between the HA^+ and HA^- clone. However, clone KF 6 had apparently more proteins of 27–30 kDa than KF 9 clone. No difference was observed if the Tx-114 phase proteins were treated with β-mercaptoethanol or were not treated with it before SDS-PAGE analysis (Fig. 3A). The main difference between the isogenic HA^+ and HA^- populations was the presence of integral membrane proteins of 51–53 kDa in clone KF 9. Immunoblotting analysis showed they were pMSA proteins recognised by mAbs 3E10, 5G6 and 4G2 (Fig. 3B). mAb 4B3 recognised the protein of about 85 kDa which were similarly synthesised in clones KF 6 and KF 9 (not shown). mAb 3E10 inhibited the HA capability of KF 9 clone cells and inhibited HAD of its colonies, which was consistent with the function of pMSA proteins in the binding of MS cells to erythrocytes (HI titer of the neat supernatant was 1:64 or 1:128). Immunoblotting using mAb

125 showed a strong reduction in staining of pMSB proteins but stronger staining of their truncated forms (of 27–30 kDa) in clone KF 6 (Fig. 3C). In immunoblotting mAb 3 also showed that clone KF 9 produced more relevant protein than clone KF 6 (Table 1). In contrast, mAbs 5 and 10 did not indicate differences in the quantity of the corresponding integral membrane proteins between the two MS clones (Table 1 and Fig. 3C). The HA^+ phenotype was associated with pMSA integral membrane proteins which were surface exposed, or at least their epitopes recognised by mAbs 3E10, 5G6 and eventually by mAb 4G2.

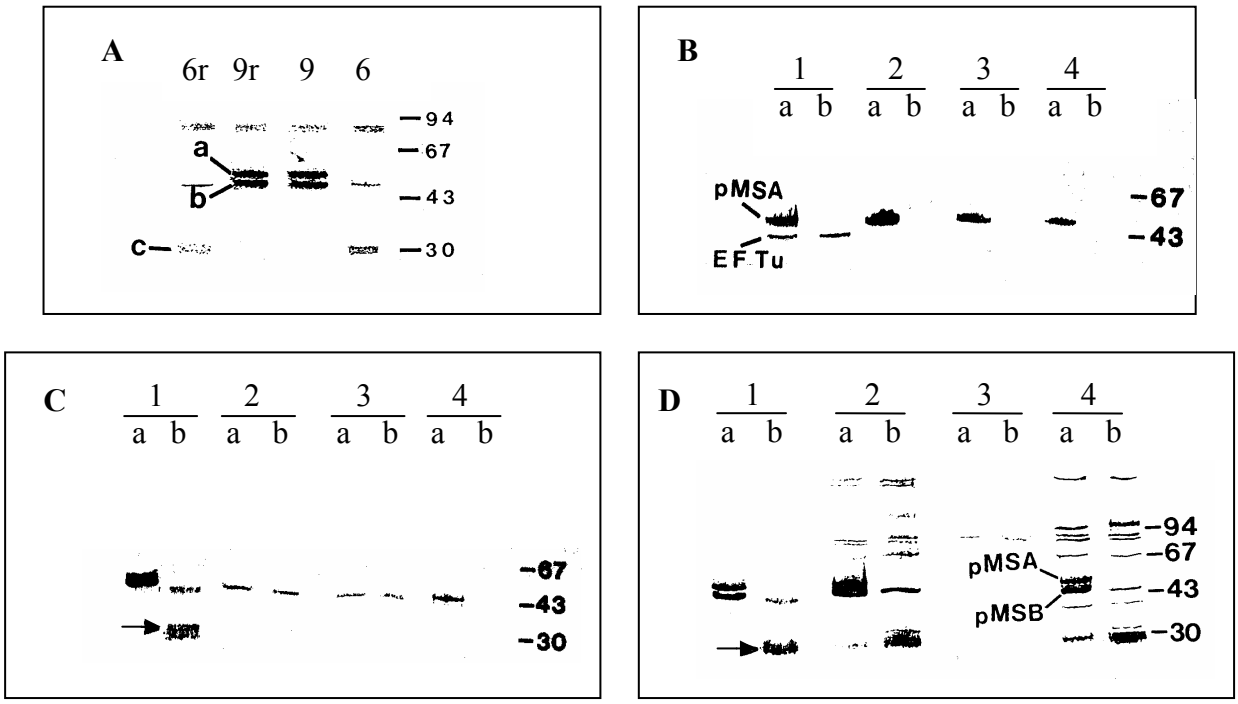

Figure 3. *M synoviae* strain ULB 925 proteins associated with haemagglutination and their immunogenicity. **Panel A**, proteins of parallel subclones with HA^+ phenotype (clone KF 9 in lanes 9) or with HA^- phenotype (clone KF 6 in lanes 6) were extracted with Tx-114, separated by SDS-PAGE and stained with Coomassie blue. The most abundant integral membrane proteins of HA^+ clone were 51–53 kDa proteins (indicated by a) and 45–47 kDa proteins (indicated by b). HA^- clone lacked 51–53 kDa proteins, had much fewer 45–47 kDa proteins, but more proteins of 27–30 kDa. There was no difference in the quantity of 80–85 kDa proteins. There was no difference if proteins were treated with β-mercaptoethanol (reducing conditions, lanes indicated with r) or were not. **Panel B**, proteins of the KF9 clone (lanes a) and KF6 clone (lanes b) were immunostained with monoclonal antibodies. 1, reaction with mAb 3E10 showed pMSA proteins only in the HA^+ clone, while the second reaction using mAb R indicated that HA^- culture synthesised a similar quantity of the EF-Tu protein; 2, reaction with mAb 3E10; 3, reaction with mAb 5G6; 4, reaction with mAb 4G2. Note the absence of pMSA proteins recognised by mAbs in the HA^- clone. In the HA^+ clone pMSA proteins partitioned almost exclusively into the Tx-114 phase. **Panel C**, the same antigens as in Panel B were immunostained by monoclonal antibodies. 1, reaction with mAb 125 (the arrow indicates a truncated 27–30 kDa $pMSB_1$); 2, reaction with mAb 5; 3, reaction with mAb 10; 4, reaction with mAb 3. Note that only mAbs 125 and 3 revealed a difference between the HA^+ and HA^- clones. **Panel D**, the same antigens as in Panel B were immunostained by 1, mAbs 3E10 and 125 to show pMSA, pMSB and truncated pMSB (indicated by the arrow); 2, by rabbit antiserum (raised against MS strain AAY-4 and diluted 1:1000) and then by mAb (to rabbit IgG) cojugated with HRP; 3, by chicken serum (diluted 1:100) taken before infection with MS; 4, by serum IgG (diluted 1:500) from a chicken which was infected with MS strain F10-2AS 2 weeks earlier. Note the antibodies showed a clear difference between the HA^+ clone proteins (the presence of pMSA and pMSB) and the HA^- clone proteins (the absence of pMSA and the transition of pMSB into truncated pMSB form of 27–30 kDa). Molecular masses (in kiloDaltons) are indicated at the right side of each Panel.

A difference in the integral membrane proteins between the HA^+ and HA^- populations was clearly recognised by chicken antibodies to MS. Chickens were experimentally infected with MS strain F10-2AS and sera were taken 2 weeks after infection. Serum IgG antibodies of a chicken with a high HI titer (1:1280) in clone KF 9 reacted with pMSA proteins, while such reaction was not observed in clone KF 6. In addition, clone KF 9 showed stronger reaction of pMSB proteins, while the truncated pMSB (27/30 kDa) reacted more intensely in clone KF 6 (Fig. 3D). On the other hand, the reactions of the protein of about 85 and 90 kDa were similar in both clones. Differences between HA^+ and HA^- phenotypes were also demonstrated by rabbit antiserum raised against MS (Fig. 3D).

Antigenic- and size- variants of pMSA and pMSB

In the parental clone KF (strain ULB 925) the transition to the HA^- phenotype (in clone KF 6) was accompanied by the appearance of truncated pMSB and disappearance of the pMSA proteins recognised by mAbs 3E10, 5G6 and 4G2. Moreover, clone KF 6 lacked integral membrane proteins of 51–53 kDa. On the other hand, in clone KF 9 subclones the transition to the HA^- phenotype was eventually different. For instance, its subclone designated KF 9/4 which lost the HA^+ phenotype and pMSA recognised by mAbs, synthesised a protein of about 50 kDa pMSB which reacted with mAb 3B4. Moreover, a subclone KF 9A5 also lost pMSA recognised by mAbs 3E10, 5G6 and 4G2. This subclone produced pMSB recognised by mAb 125 as a protein of about 45–47 kDa, and traces of tpMSB of 27–30 kDa. In IIPA an alternative staining of MS colonies with either mAb 3B4 or with mAb 3E10 was noticed (see above). This suggested that pMSA proteins defined by mAb 3E10 were not coexpressed with the pMSB antigenic variant detected by mAb 3B4.

Antigenic differences concerning pMSA and pMSB proteins were also observed in other MS strains. mAb 50 which in MS strain WVU-1853 defines MSPB (a homologue of pMSB) reacted in IIPA with HAD^+ colonies of MS strains K2581 and F10-2AS, but not with those of strains ULB 925, K1723, PAA-2 and FMT. However, in immunoblotting of HA^+ cultures of strains K2581 and F 10-2AS, mAb 50 reacted strongly with proteins of about 43 kDa. They were a few kDa shorter than the pMSB proteins of MS strains ULB 925 and WVU-1853. However, in MS strains K2581 and F 10-2AS proteins detected by mAb 50 could not be distinguished by immunoblotting from pMSB proteins detected by mAb 125 (Fig. 4B). Overall, in different MS strains pMSB proteins with epitopes for mAb 125 were synthesised rather constantly, although they sometimes appeared as truncated forms, i.e. tpMSB. On the other hand, proteins detected by mAbs 3, 50 or 5F7 showed evident phase-variation and eventually quantal-variation in expression even in isogenic lineages of appropriate MS strains. The diversity of pMSA proteins was even more apparent. mAbs 3E10, 5G6 and 4G2 reacted with pMSA proteins of MS strains AAY-4, ULB 925, WVU-1853, F10-2AS, FMT, K2581 and several MS field strains. However, in numerous strains including F10-2AS, WVU-1853, K2581 and FMT reference strains, their HAD^+ and HA^+ phenotypes were not correlated with the expression of the proteins detected by mAbs to pMSA proteins. In addition, a considerable size-variation of the proteins recognised by chicken antibodies was observed among different MS strains. Serum IgG antibodies of a chicken experimentally infected with strain F10-2AS recognised in clone KF 9 pMSA (51–53 kDa), but in strain K1723 (clone K23) they showed the strongest reaction with VLHA protein of about 90 kDa (Fig. 4C). Immunoblotting reactions of strains K2581, FMT and F10-2AS indicated in these strains shorter pMSB proteins (Fig. 4C and data not shown).

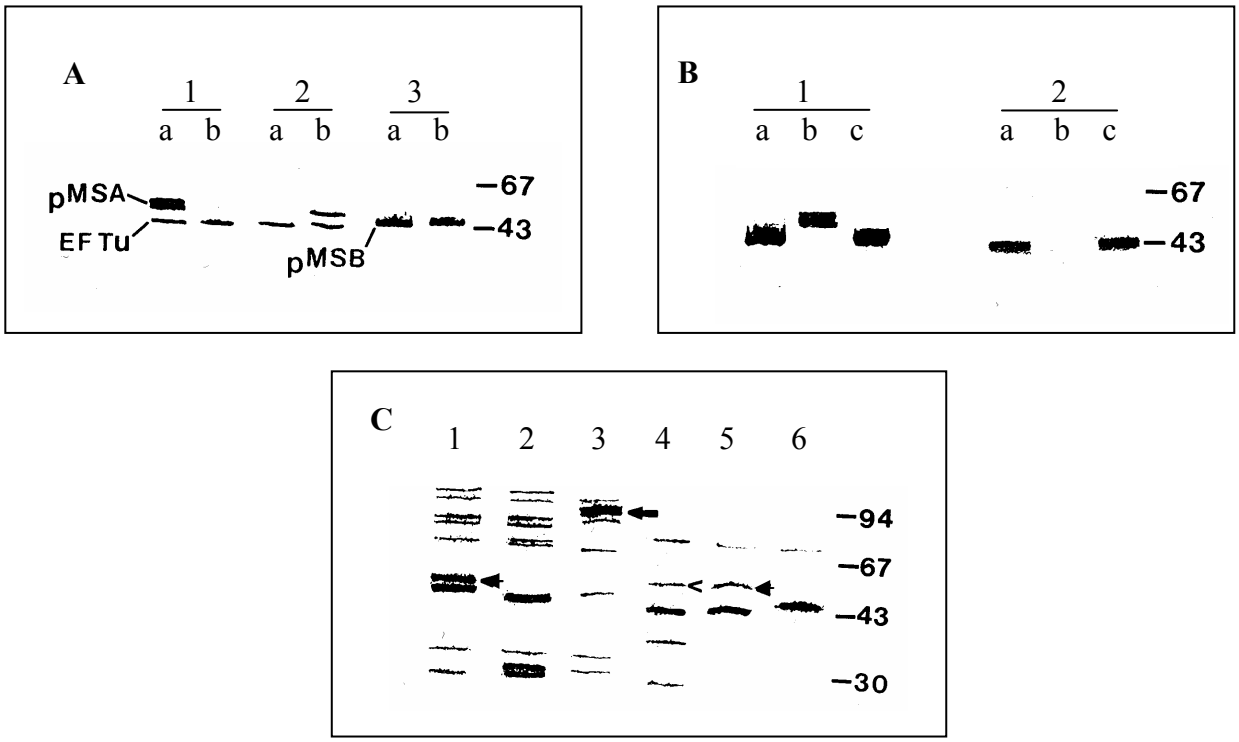

Figure 4. Antigenic and size-variation in *M. synoviae*. **Panel A**, strain ULB 925 KF 9 (lanes a) and its subclone KF 9A5 were immunoblotted with mAbs. 1, reaction with pooled mAbs (3E10 and R) showed pMSA and EF-Tu proteins; 2, reaction with pooled mAbs (3B4 and R) indicated that clone KF 9A5 lacking $pMSA_1$ synthesised an antigenic variant of pMSB (50 kDa) recognised by mAb 3B4; 3, reaction with mAb 125 did not indicate any difference in pMSB proteins in the two clones. **Panel B**, immunoblot of HA^+ cultures of MS strains K2581 (lanes a), ULB 925 (KF 9, lanes b) and F10-2AS (K8, lanes c) with monoclonal antibodies. 1, reaction with mAb 125 showed a few kDa longer pMSB in ULB 925 strain; 2, reaction with mAb 50 showed the absence of the relevant protein in ULB 925 strain but strong staining of about 41–43 kDa proteins in two other MS strains. **Panel C**, immunoblot of chicken serum IgG (diluted 1:500, see Figure 3, Panel D) with proteins of 6 MS strains. MS strains are in lanes: 1, ULB 925 (clone KF 9); 2, WVU-1853 (HA^- clone); 3, K1723 (HA^+ clone K23); 4, FMT (HA^+ culture); 5, K2581 (HA^+ culture) and 6, F10-2AS (HA^+ clone K8). The arrowheads indicate pMSA (lane 1) or putative pMSA-proteins (lanes 4 and 5). Note the lacking pMSA and pMSB proteins (with molecular weight from 40 to 55 kDa) in strain K1723 where the strongest reaction occurred with a protein of about 90 kDa (indicated by an arrow) which is an uncleaved VLHA protein. Molecular masses of proteins (in kiloDaltons) are indicated for all panels on the right hand side.

DISCUSSION

Mycoplasmas use adaptive variation as a survival strategy in their immunocompetent hosts. They lack a cell wall and therefore their surface membrane proteins are of crucial importance in interactions with host environments. Certain membrane proteins are transporters of the essential nutrients which mycoplasmas need to obtain from the in-host environment, while others mediate attachment to host cell receptors and are usually highly immunogenic. Despite the apparent paucity of recognisable regulatory components that modulate and optimise gene expression according to environmental changes, mycoplasmas colonise diverse host niches and evade host immune responses, because they are capable of changing the structure and/or expression of key surface components (Zhang and Wise, 1997; Razin *et al.,* 1998).

Previous studies have shown that MS agglutinates erythrocytes through neuraminic acid receptors and that HA capability varies between different clones of a given MS strain (Manchee and Taylor-Robinson, 1969; Rhoades, 1985). MS attachment to erythrocytes may not be exactly

the same as its attachment to the cells on the surface of the organs which it colonises. However, HA^+ clones of MS AAY-4 strain are more capable of inducing acute synovitis than are HA^- clones (Narat *et al.,* 1998). Identification of the *vlhA* gene encoding MS haemagglutinin gave a better insight into its molecular structure (Noormohammadi *et al.,* 1998). It was shown that in the majority of MS strains examined, the *vlhA* gene product undergoes cleavage and we recently identified a putative cleavage site of VLHA in clone KF 9 of MS strain ULB 925 (Benčina *et al.,* 1999). A previous study using type strain WVU-1853 already indicated phase-variable expression of MSPB and MSPA and their association with the HAD^+ phenotype (Noormohammadi *et al.,* 1997). The authors reported that cloning of uniformly HAD^+ colonies yielded cultures in which some 65 % of the colonies were HAD^+. The present study showed that cloning of uniformly HAD^+ colonies of strain ULB 925 yielded up to 99 % HAD^+ progeny population and this was also reported by Rhoades for MS strains WVU-1853 and Neb-3S (Rhoades, 1985). He also reported that HA characteristic were stable "*in vitro*" but not "*in vivo*". Data from this study clearly show that the HA phenotype is not absolutely stable, though a great majority of the subpopulation retains the parental HA^+ phenotype. At present in the HA^- population the genetic basis generating a truncated haemagglutinin (i.e. truncated pMSB) and the absence of pMSA is not clear. In several *Mycoplasma* species, including those which infect humans (*M, pneumoniae*, *M. hominis* and *M. fermentans*), reversible mutations occur in genes encoding adhesins or variable surface lipoproteins. This results in a frameshift and generation of a termination codon, causing the premature termination of protein translation (Su *et al.,* 1989; Theiss and Wise, 1997; Zhang and Wise, 1997). Recently, it has been proposed that recombinations of the *vlhA* gene with one of the pseudogenes generate antigenic variants of MSPB and MSPA proteins (Noormohammadi *et al*., 200). Our recent study showed that site-specific *vlhA* gene recombinations generate MSPB variants recognised either by mAB 50 or mAb 3B4 (Benčina *et al*., 2000). Our previous amino acid sequencing of the pMSA proteins of clone KF 9 culture yielded three different sequences (Benčina *et al.,* 1999). This indicates that at least three different *vlhA* sequences were expressed in that culture. In *M. gallisepticum* strain S6 two different members of the pMGA gene family (i.e. pMGA1.1 and 1.9) can be expressed at the same time (Markham *et al.,* 1998; Glew *et al.,* 1998). It seems that the pMGA and *vlhA* gene families have arisen by the horizontal gene transfer, possibly as a result of a shared habitat (Noormohammadi *et al.,* 1998). We have already reported that mAb recognising MS proteins of 30 and 45–50 kDa (i.e. mAb 125) showed in IIPA sectorial staining of about 5 % of the colonies of *M. gallisepticum* S6 strain (Benčina *et al.,* 1994a). At present this reaction can be explained by an epitope shared by MS VLHA protein and *M. gallisepticum* pMGA 1.7 protein (Noormohammadi *et al*., 1998). It seems that mAb 125, which reacted consistently with proteins (pMSB or MSPB) of all MS strains examined, recognises an epitope in the conserved N-terminal part of the pMSB protein (Benčina *et al.,* 2000). The 5'-end of the *vlhA* gene seems to be rather conserved in MS strains, at least in those we have examined. On the other hand, in the region encoding repeats rich with prolines (Noormohammadi *et al.,* 1998) the number of repeats varies. In this region *vlhA* genes of MS strains K2581, F10 2AS and FMT lack 15–19 amino acids in comparison with the published sequence of the WVU-1853 strain (Noormohammadi *et al.,* 1998). These deletions are consistent with the finding that their pMSB proteins are a few kDa shorter than pMSB of MS WVU-1853 or ULB 925 (KF 9) in immunoblotting with mAb 125 (see Fig. 4B and C). Previous studies have already shown a considerable size- variation of MSPB proteins in MS strains (Noormohammadi *et al.,* 1997) and of proteins which might be pMSB proteins (Avakian *et al.,* 1992). Our analyses showed that deletions or insertions in the *vlhA* regions encoding proline-rich repeats are the molecular bases for the size-variation of pMSB proteins (Benčina *et al*., 2000). We confirmed that MS strain K1723 produced an uncleaved VLHA haemagglutinin, as has been already reported (Noormohammadi *et al*. 1997, 1998). Its clone K23 which synthesised an abundant VLHA protein of about 90 kDa had a high HA titer

i.e. 2 x 10^4. Moreover, the 90 kDa protein was strongly recognised by chicken antibodies to pMSB and pMSA of MS strains AAY-4 and ULB 925 (Narat *et al.,* 1998).

It has been reported that MS strain proteins of 80 and 90 kDa bind chicken IgG via their Fc part (Lauerman *et al.,* 1993). We did not observe such binding, at least not at the level which would disturb the estimation of a positive reaction with chicken sera containing IgG antibodies to MS. In certain strains (clones) (e.g. of F10-2AS) preinfection sera eventually gave weak reactions of 80 or 90 kDa proteins, but the reactions with sera containing IgG antibodies were much stronger (see Fig. 3D).

With the reference to the variable expression of *M. gallisepticum* proteins we already recommended that antigens and vaccines should be prepared from cultures with defined antigenic determinants in order to achieve consistent and reproducible results (Benčina *et al.,* 1994b). The same is valid for MS. Indeed, we observed that one special batch of the MS antigen (strain WVU-1853) produced for the rapid serum plate agglutination gave unusually strong cross-reactions with chicken sera containing only antibodies to *M. gallisepticum*. This antigen contained protein of about 50 kDa recognised by chicken IgG to *M. gallisepticum*. This cross-reactive protein was not found in other antigen batches from the same production.

In conclusion, our data reinforce previous observations that MS haemagglutinin(s) are capable of phase-, size- and antigenic variation. This is very important because MS haemagglutinins play a major role during infection, including host immune response and seem to be associated with MS pathogenicity.

POVZETEK

Mikoplazme izrabljajo mehanizem spremenljivega izražanja nekaterih beljakovin kot strategijo za preživetje v imunokompetentnem gostitelju. Med najpomembnejše spremenljivo izražene in imunogene beljakovine sodijo adhezini, ki so nujni za pritrjevanje na epitelne celice tkiva in so vpleteni v mehanizem pritrjevanja na eritrocite in pri njihovi hemaglutinaciji. Predhodne raziskave so potrdile, da hemaglutinacijsko pozitivni (HA^+) kloni bistveno bolj vzbujajo infekciozni sinovitis pri preskusnih živalih kot hemaglutinacijsko negativni (HA^-) kloni, kar kaže na to, da so hemadhezini vpleteni tudi v razvoj bolezni, ki jih mikoplazme povzročajo.

Hemaglutinin VLHA je kodiran na *vlhA* genu in se izraža spremenljivo v molekulski masi kot tudi v fazi, kar pogojuje različne fenotipe, HA^+ ali HA^-. Značilna za HA^+ klone je prisotnost obeh podenot VLHA beljakovine: pMSA, ki reagira z mAb 3E10 in ima molekulsko maso 51 kda ali 53 kDa in pMSB, ki reagira z mAb 125 in ima molekulsko maso 47 kDa ali 49 kDa. Za HA^- klone je značilno, da podenote pMSA ne izražajo, podenota pMSB pa ima nižjo molekulsko maso, vendar še vedno reagira z mAb 125. Naše analize dokazujejo, da so delecije ali insercije v regiji, ki vsebuje ponovitve bogate s prolini v *vlhA* genu, odgovorne za razlike v molekulski masi različic pMSB podenote.

Glede na vedno nove potrditve o spremenljivem izražanju imunogenih membranskih beljakovin pri mikoplazmah je pomembno poznati mehanizme, ki povzročajo te spremembe. Še posebej je to pomembno za tiste beljakovine, ki se uporabljajo za pripravo antigenov za testiranje, in za tiste, ki se uporabljajo za pripravo cepiv. V obeh primerih morajo biti namreč beljakovine vedno enake, če naj zagotovijo ponovljive in zanesljive rezultate preskusov in uspešno cepljenje.

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