A newly identified immunodominant membrane protein (pMB67) involved in *Mycoplasma bovis* surface antigenic variation

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***Mycoplasma bovis* is a bovine pathogen able to cause systemic disease. It possesses a series of prominent, structurally related yet clearly distinguishable membrane lipoproteins on the cell surface. These variable surface proteins (Vsps) undergo highly dynamic and spontaneous changes in size and expression and are key immunogenic components. They may play a critical role as mediators of adherence to host cells and in escaping immune destruction. In this report, we define a novel, Vsp-unrelated membrane protein also associated with *M. bovis* surface antigenic variation. This protein has an apparent molecular mass of 67 000 Da in the type strain PG45 and was designated pMB67. Immunological and biochemical characterization of pMB67 demonstrated that it: (i) contains a specific epitope, (ii) is not modified by lipid but does contain cysteine, (iii) does not contain a Vsp-like repetitive periodic protein structure, (iv) is a predominant antigen recognized during *M. bovis* infections, (v) undergoes a high rate of phase variation *in vitro* and (vi) is size-variable. These results showed that *M. bovis* employs two types of specialized membrane proteins for surface diversification. The pMB67 protein may be useful in diagnostic assays and as a vaccine component.**

Keywords: *Mycoplasma bovis*, variable membrane proteins, immunodominant antigens, surface antigenic variation, phase variation

INTRODUCTION

Mycoplasma bovis is one of the most important pathogenic bovine mycoplasmas. In contrast to many other species, which colonize the mucosal surfaces and establish localized chronic infections, this organism has the potential to cross mucosal barriers (Howard *et al.*, 1987) and cause systemic infections after entering the bloodstream. This ability to disseminate systemically may account for the isolation of *M. bovis* from multiple body sites (Ross,

1985) and its association with a variety of clinical manifestations, such as mastitis (Brown *et al.*, 1990), arthritis (Hjerpe & Knight, 1972), pneumonia (Gourlay *et al.*, 1989), subcutaneous abscesses (Kinde *et al.*, 1993), meningitis (Stipkovits *et al.*, 1993) and infertility (Ruhnke, 1994). Despite attempts at eradication, *M. bovis* infections are prevalent worldwide with a particularly high incidence in Europe and North America (Pfützner, 1990; ter Laak *et al.*, 1992; Nicolet, 1994) and present a continuing significant problem that is economically damaging to the dairy and meat industry. Failure to prevent and control *M. bovis*-induced diseases is due to the ineffectiveness of antibiotic therapy and, in particular, the lack of vaccines for economically feasible prophylactic immunization and of specific and sensitive commercially available diagnostic tools (Pfützner, 1990; ter Laak *et al.*, 1992).

An understanding of the pathogenesis of systemic disease caused by *M. bovis* is necessary for rational vaccine design.

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Abbreviations: pAb, polyclonal antibody; TX-114, Triton X-114; Vsp(s), variable surface protein(s) (lipoprotein family).

Previous work has focussed on the identification and structural analysis of *M. bovis* surface antigens which are prominent targets of the bovine immune response and may influence key host interactions. This has resulted in the identification of a family of multiple membrane surface lipoproteins with three defined members (VspA, VspB and VspC) which (i) contain cross-reactive epitopes, i.e. can be recognized by cross-reactive monoclonal and polyclonal antibodies (mAbs and pAbs, respectively) (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994); (ii) undergo a high rate of size and phase variation *in vitro* (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994); (iii) are encoded by a family of *vsp* genes (I. Lysnyansky, R. Rosengarten & D. Yogeve, unpublished results); (iv) are expressed by invasive *M. bovis* isolates, for instance, those from joints and urine (Rosengarten *et al.*, 1994); (v) are among the predominant antigens recognized during infection and disease (Rosengarten *et al.*, 1994); (vi) may be involved in *M. bovis* adhesion to host cells (Sachse *et al.*, 1996); and (vii) may have diagnostic and immunoprophylactic value despite their variation (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994).

Recent preliminary observations have suggested that *M. bovis* may utilize more than one antigenic variation system for surface diversification (Poumarat *et al.*, 1994); however, the biochemical nature and cellular location of these components have not been extensively defined. The present study confirms these observations and demonstrates that an abundant membrane surface protein that is antigenically and structurally unrelated to the Vsp family contributes to surface variation of *M. bovis*. This protein may act as a major immunological target during infection and may prove useful in serodiagnosis, epidemiological studies and vaccine development.

METHODS

Mycoplasmas and culture conditions. *M. bovis* strains PG45 (type strain), D857, D1499, D1115 and D1511 have been previously described (Rosengarten *et al.*, 1994); strains D857, D1499, D1115 and D1511 are field isolates which have not been filter-cloned (Rosengarten *et al.*, 1994). All strains were cultured in the medium of Rosengarten *et al.* (1994). Subclones of type strain PG45 were obtained and screened for expression of surface antigens as described elsewhere (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994).

Antibodies. Two mAbs directed toward distinct *M. bovis* membrane surface proteins were used in this study: mAb 1E5 is an immunoglobulin (Ig) M(κ) isotype and recognizes a surface epitope on at least three members of the variable integral membrane surface lipoprotein (Vsp) family of *M. bovis* (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994); mAb I₂ (Poumarat *et al.*, 1994) was kindly provided by Vétroquinol Biotechnologie and found here to be an IgG1(κ) isotype. Other antibodies used were pAb anti-D490, a rabbit hyperimmune serum prepared against *M. bovis* strain D490, and serum sample D74, from a cow with naturally induced *M. bovis* arthritis (Rosengarten *et al.*, 1994). For immunostaining of blots, antibodies were diluted 1:50 (bovine serum D74), 1:100 (mAb 1E5) or 1:1000 (mAb I₂; pAb anti-D490) in phosphate-buffered

saline (PBS; 2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.0 mM NaH₂PO₄, pH 7.2) for colony blots or PBS containing 0.1% (v/v) Tween 20 (PBS-T) for Western blots.

Mycoplasma antigen analysis. The procedures for Triton X-114 (TX-114; Boehringer Mannheim) phase fractionation, metabolic labelling with [³⁵S]cysteine and [³H]palmitate, SDS-PAGE, fluorography and Western blotting of mycoplasma proteins were as previously described (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994). For SDS-PAGE, samples were treated at 100 °C for 5 min under reducing conditions and loaded onto 9% (w/v) polyacrylamide gels containing 3% (w/v) urea. Gels containing radiolabelled proteins were fluorographed for 7–10 weeks at –80 °C. For Western blot analysis of unlabelled or [³⁵S]cysteine-labelled phase-fractionated or whole-cell proteins, blots were incubated for 1 h at 60 °C, blocked overnight at 4 °C with PBS containing 3% (w/v) bovine serum albumin, washed three times in PBS-T and then incubated overnight at 4 °C with the primary antibodies. After washing with PBS-T, blots were incubated for at least 2 h at room temperature in peroxidase-conjugated secondary antibodies (goat; Nordic) against mouse IgM, mouse IgG or rabbit immunoglobulins, each diluted 1:1000 in PBS-T. After repeated washing in PBS, blots were developed using 4-chloro-1-naphthol. Blots containing [³⁵S]cysteine-labelled proteins were autoradiographed for 10 weeks by direct exposure to films at –80 °C. To determine the surface location and orientation of pMB67, intact organisms from fresh broth cultures were treated with various amounts of trypsin or carboxypeptidase Y (which has a strict requirement for a free C-terminal residue) as previously described (Behrens *et al.*, 1994) and the digestion products analysed in Western blots as described above.

Colony immunoblotting. The variable expression of pMB67 and Vsps within *M. bovis* clonal populations was detected in colony immunoblots which were prepared as previously described (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994). To distinguish among clonal variants expressing pMB67 or Vsps (VspA, VspB and VspC in any combination) only, and those co-expressing pMB67 and Vsps, colony blots were consecutively immunostained with mAbs I₂ and 1E5 using the chromogenic substrates 4-chloro-1-naphthol, which gives a blue colour reaction (for detection of pMB67), and *o*-dianisidine, which gives a brown colour reaction (for detection of Vsps). Colonies co-expressing pMB67 and Vsps showed a mixed colour (brown-blue).

RESULTS

Identification of a prominent 67 kDa antigen (pMB67) in *M. bovis* type strain PG45 that has different-sized counterparts in other *M. bovis* strains

Identical blots of whole-cell proteins from broth-grown *M. bovis* strain PG45 and four additional strains were separately immunostained with the Vsp-specific mAb 1E5 (Fig. 1a) and mAb I₂ (Fig. 1b). The results obtained with mAb I₂ identified a set of antigens exhibiting size polymorphism among the five strains analysed, with up to three differently sized multiple antigens within a single strain (Fig. 1b, lanes 1–3), as recently reported (Poumarat *et al.*, 1994). Close comparison of the two immunoblots prepared using mAb 1E5 and mAb I₂ revealed striking differences in the migration patterns of the antigens

Fig. 1. Identification of a 67 kDa strain-variant *M. bovis* antigen, pMB67, that is not recognized by Vsp-specific mAb 1E5. (a, b) Western immunoblot analysis of whole-organism proteins from *M. bovis* type strain PG45 (lanes 1) and strains D857 (lanes 2), D1499 (lanes 3), D1115 (lanes 4) and D1511 (lanes 5) with mAb 1E5 (a) or I₂ (b). Each lane contains proteins from equal quantities (20 µl) of mid-exponential-phase cultures. The positions of VspA65 and VspB46 (a) and pMB67 (b) as expressed by the type strain are indicated on the left. The positions and relative molecular masses (in kDa) of as yet undefined Vsps (a) and pMB67-related size-variant antigens (b) expressed by the other four strains are indicated on the right. (c) Equivalent amounts of total cell protein [13 (lanes 1–3) and 6 (lanes 4–6) µl culture per lane] from three clonal variants of strain PG45 (clone 26, lanes 1 and 4; clone 27, lanes 2 and 5; clone 28, lanes 3 and 6) were run in two sets of adjacent lanes and the resulting blot was divided and immunostained with mAb I₂ (lanes 1–3) and mAb 1E5 (lanes 4–6). The positions of pMB67, VspA65 and VspB46 are indicated.

recognized; indeed, none of the five strain- and size-variant antigens recognized by mAb I₂ (Fig. 1b) could be identified as mAb 1E5-binding Vsp (Fig. 1a). Although a 48 kDa antigen of *M. bovis* D857 (Fig. 1b, lane 2) and a 40 kDa antigen of *M. bovis* D1499 (Fig. 1b, lane 3) recognized by mAb I₂ co-migrated with a Vsp product (Fig. 1a, lanes 2 and 3, respectively) in these strains, the corresponding bands were not identical, since the distribution of these two mAb-I₂-defined antigens among the five strains analysed (Fig. 1b) did not correspond to the strain distribution of the two similar-sized Vsps (Fig. 1a).

The most prominent antigen reacting with mAb I₂ was identified in the type strain PG45 (Fig. 1b, lane 1). This antigen was confirmed to be a protein by its susceptibility to trypsin degradation as well as its labelling properties (see below) and was designated by its apparent molecular mass in kDa as pMB67. To further confirm that this antigen was a distinct product and not a size variant form of VspA (compare Fig. 1a and b, lanes 1), equivalent amounts of total protein from three clonal variants of type strain PG45 were subjected in replicate to SDS-PAGE, and subsequent blots (Fig. 1c) were immunostained with mAb I₂ (lanes 1–3), mAb 1E5 (lanes 4–6) or pAb anti-D490 (data not shown). The resolution provided by these immunoblots allowed unambiguous identification of pMB67 as a discrete translational product with a reaction profile (binding of mAb I₂ only) distinct from that of VspA (binding of mAb 1E5 and pAb anti-D490; see also Rosengarten *et al.*, 1994). Also apparent was the non-coordinate expression of pMB67, VspA and VspB, since all three clonal variants displayed different levels of

expression of VspA or VspB, while pMB67 was expressed in similar quantities.

pMB67 is a surface-exposed, membrane-associated nonlipoprotein with a free C-terminus

The localization and topology of pMB67 on the external surface of *M. bovis* was examined, using mAb I₂ to monitor its susceptibility to trypsin- and carboxypeptidase-Y-digestion in intact mycoplasma cells prepared from mid-exponential-phase cultures. Western blots of these preparations showed a gradual reduction of staining with increasing enzyme concentration (Fig. 2a and b), without generation of smaller epitope-bearing degradation products. In contrast, digestion of VspA, VspB and VspC with carboxypeptidase Y gave a series of products of varying size, which could be immunostained with mAb 1E5, and following digestion with trypsin the smallest immunoreactive fragments ranged from 41 to 45 kDa (Behrens *et al.*, 1994). These results established that (i) pMB67 is located on the organism's surface, (ii) its C-terminus has a free carboxyl residue that is accessible on the surface, and (iii) its enzyme digestion profiles are clearly distinguishable from those of Vsps.

Although Poumarat *et al.* (1994) indicated that the strain-variant antigenic components recognized by mAb I₂ are associated with the membrane, the biochemical nature and location of these antigens have not been extensively defined. Using procedures previously described (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994), we were able to provisionally classify pMB67 as an integral

Fig. 2. Biochemical characteristics of pMB67. (a, b) Susceptibility of pMB67 to trypsin and carboxypeptidase Y treatment of intact cells. Fresh broth-grown cells (25 μ l culture per sample) of a clonal variant of *M. bovis* PG45 incubated with increasing concentrations of trypsin (a) or carboxypeptidase Y (b) were subjected to SDS-PAGE and the subsequent Western blots immunostained with mAb I₂. Lanes 1 represent organisms incubated in enzyme buffer, lanes 2–6 represent organisms treated, respectively, with 5, 10, 20, 50 or 100 μ g trypsin ml⁻¹ (a), or with 40, 100, 150, 200 or 400 μ g carboxypeptidase Y ml⁻¹ (b). Restaining of the blots with pAb anti-D490 showed that the cytoplasmic protein p41 (Rosengarten *et al.*, 1994) was not degraded, so confirming that cells remained intact during digestion (data not shown). (c) TX-114 phase partitioning characteristics of pMB67. Broth-cultured organisms of a clonal variant of *M. bovis* PG45 were phase-fractionated with TX-114, and whole-organism proteins (lane 1), TX-114 phase proteins (lane 2) or aqueous phase proteins (lane 3) were separated by SDS-PAGE and immunoblotted with mAb I₂. (d, e) Metabolic labelling characteristics of pMB67. TX-114 phase proteins metabolically labelled with [³⁵S]cysteine (d) or [³H]palmitate (e) from selected clonal isolates of *M. bovis* PG45 expressing pMB67 (lanes 1), VspB42 (lanes 2) or VspA63 (lanes 3) were separated by SDS-PAGE and analysed by fluorography. Identical populations are represented in the corresponding lanes. Samples in panel (c) contained whole-organism (lane 1) or phase-fractionated proteins (lanes 2 and 3) from 10 (lane 1), 25 (lane 2) or 180 (lane 3) μ l mid-exponential-phase cultures. Each lane in panels (d) and (e) represents TX-114 phase proteins from 1800 μ l mid-exponential-phase cultures. pMB67, VspA63 and VspB42 visualized by immunostaining and/or radiolabelling are indicated. The autoradiographic signal of the VspB42 band in panel (e) (lane 2) is weak because of the limited quantity of total protein present.

membrane protein with characteristics of a non-lipoprotein. Firstly, the clear separation of pMB67 as an amphiphilic product into the detergent phase during TX-114 phase fractionation of whole organisms allowed its identification as a putative integral membrane protein (Fig. 2c). And secondly, comparison of TX-114 phase proteins from [³⁵S]cysteine- or [³H]palmitate-labelled organisms, visualized by fluorography, revealed that pMB67 contained cysteine (Fig. 2d, lane 1) but not fatty acid (Fig. 2e, lane 1). In contrast, a 63 kDa size variant of VspA (Fig. 2d and e, lanes 3) and a 42 kDa size variant of VspB (Fig. 2d and e, lanes 2) which served as control markers, were labelled with both compounds (Behrens *et*

al., 1994). This was rigorously confirmed by immunostaining Western blots of radiolabelled TX-114 phase proteins, followed by autoradiography to precisely align immunostained bands with their radiolabelled counterparts (data not shown).

pMB67 undergoes high-frequency *in vitro* phase variation and is size-variable

Western blot immunoscreening with pMB67-specific mAb I₂ of a series of colonies, which were randomly selected from a cloned subpopulation, revealed a striking heterogeneity in the relative amount of pMB67 present in

Fig. 3. Variation in expression and size of pMB67. (a) Different expression levels of pMB67 among broth-cultured randomly-selected isolates of a clonal subpopulation of *M. bovis* PG45 (clone 26, see Fig. 1c, lanes 1 and 4). Whole-organism proteins from 25 μ l of mid-exponential-phase cultures were separated by SDS-PAGE and immunoblotted with mAb I₂. The blot was restained with pAb anti-D490 recognizing invariant protein p41 (Rosengarten *et al.*, 1994), which served as a control to ensure that similar amounts of organisms were loaded per sample (data not shown). (b) Colony immunoblot of a clonal population of *M. bovis* PG45 demonstrating phase variation in expression of pMB67. The blot was immunostained with Vsp-specific mAb 1E5 using α -dianisidine as chromogenic substrate giving a light brown colour reaction and restained with mAb I₂ to pMB67 using 4-chloro-1-naphthol as chromogenic substrate giving a dark blue colour reaction. A representative portion of the blot is shown. One colony variant displaying the pMB67⁺ Vsp⁺ phenotype and one colony variant displaying the pMB67⁻ Vsp⁺ phenotype are indicated. Two colonies and one colony sector showing the pMB67⁻ Vsp⁻ phenotype (negative reaction) are indicated by arrowheads. (c) Western blot analysis of whole-organism proteins from a clonal lineage of *M. bovis* PG45 with mAb I₂ showing phase variation in expression of pMB67 ('phase on' $\rightarrow$ 'phase off' $\rightarrow$ 'phase on') corresponding to the selected colony phenotype. Phase transitions are indicated by arrows above lanes. Each lane contains proteins from 20 μ l mid-exponential-phase cultures. (d) Western blot analysis of total proteins from three randomly selected clonal subpopulations of *M. bovis* PG45 clone 26 (lanes 1–3) with mAb I₂, one of which (lane 3) expressed a 63 kDa size variant of pMB67. Progeny populations (lanes 4–7) of this clonal variant showed stable expression of the 63 kDa size variant (lanes 4, 6 and 7) or reversion to the original 67 kDa form of pMB67 (lane 5). The position and size of the smaller pMB67 is indicated by the arrow on the right. Each lane contains proteins from 25 μ l mid-exponential-phase cultures. The bands of pMB67 in lane 5 and of the smaller 63 kDa variant are less prominent because of the limited quantity of total protein present in these lanes.

the broth-cultured populations analysed (Fig. 3a). For example, while the clonal variants represented in lanes 4, 5, 7 and 9 showed a strong relative staining intensity of this protein, those represented in lanes 1–3 showed a much weaker staining of the corresponding band, and in one clonal variant (lane 6), pMB67 was not detectable. pMB67 expression variants were plated and analysed by colony immunoblotting with mAb I₂ enabling identification of both pMB67⁺ and pMB67⁻ colony variants within the various populations analysed. One example of this colony immunoblot analysis demonstrating a mixed population of pMB67⁺ and pMB67⁻ variants is illustrated in Fig. 3(b). Restaining of these colony blots with Vsp-specific mAb 1E5 allowed the identification of four different mAb-binding phenotypes (three of which are shown in Fig. 3b), and further confirmed (see above) that the expression of pMB67 is not coordinately linked with the expression of VspA, VspB or VspC. The following observations unequivocally established that pMB67 was indeed subject to high-frequency (10^{-3} to 10^{-4} per generation) reversible phase switching. Firstly, further propagation and replating of colonies scored as pMB67⁺ or pMB67⁻ revealed that a small fraction (0.7–8.0%) of progeny colonies always showed the alternate (negative or positive) phenotype (data not shown). Secondly, these phase variants gave rise in turn to revertants expressing the original phenotype (data not shown). Thirdly, colonies frequently displayed a sectorized pattern of immunostaining, indicating switching between the pMB67⁺ and pMB67⁻ phenotype during colonial growth (Fig. 3b). And finally, Western immunoblot analysis of clonal lineages selected by colony immunoblots and representing successive generations clearly showed oscillating expression of pMB67, for example (as shown in Fig. 3c), from 'phase on' (lane 1) to 'phase off' (lane 2) and again to 'phase on' (lane 3).

Another feature of pMB67, also characteristic of the Vsps (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994), was size variation. One example of this size variability is illustrated in Fig. 3(d), in which a 63 kDa size variant of pMB67 (lane 3) is shown to spontaneously revert to the original size form of 67 kDa (lane 5). However, in contrast to Vsp size variants (Behrens *et al.*, 1994), size variants of pMB67 were detected at a very low frequency. In addition, simultaneous appearance of a number of size variants in ladder-like patterns, as reported for the Vsp system (Behrens *et al.*, 1994), was never observed, even in heavily loaded samples (see Fig. 3d, lane 1).

pMB67 is a major immunogen of *M. bovis* recognized during infection and disease

The finding that pMB67 was a major surface antigen of *M. bovis* raised the possibility that it may be an important immunogenic component during infection and disease, as recently demonstrated for the Vsps (Rosengarten *et al.*, 1994). To determine whether this was the case, serum from a cow with naturally induced *M. bovis* arthritis (Rosengarten *et al.*, 1994) was used (Fig. 4c) in conjunction

Fig. 4. Recognition of pMB67 by serum antibodies from an infected cow. Total proteins from three clonal variants of *M. bovis* PG45 expressing VspC77 (lanes 1), VspA63 plus VspB46 (lanes 2) or pMB67 (lanes 3) were subjected to SDS-PAGE and immunostained with mAb 1E5 (a), mAb I₂ (b) or bovine serum D74 from a clinical case of *M. bovis* arthritis (c). The lanes contain proteins from 10 (a and c, lanes 1 and 2), 20 (c, lane 3) or 25 (b) μ l of mid-exponential-phase cultures. In panel (c) lane 3, the pMB67 band is faint and there is virtually no background staining because of the limited quantity of total protein present.

with mAb 1E5 (Fig. 4a) and mAb I₂ (Fig. 4b) to probe Western blots of whole-cell proteins from three clonal variants expressing a set of Vsps (lanes 1 and 2) and pMB67 (lanes 3). Comparison of the three immunoblots which were prepared from the same gel showed precise alignment of the individual mAb-defined antigens, i.e. VspA, VspB, VspC and pMB67 (Fig. 4a and b), with the most prominent bands immunostained by the bovine serum antibodies (Fig. 4c). Western immunoblot analysis of other serum samples from *M. bovis*-infected cattle confirmed these data (not shown). These results demonstrated that pMB67 is among the major *M. bovis* components that induce antibody during natural infection and disease.

DISCUSSION

The identification of *M. bovis* surface structures potentially involved in immune evasion, colonization and invasion is of fundamental importance for understanding the pathogenesis of *M. bovis* systemic infections and for developing effective immunological intervention strategies. In previous studies such surface components, which may play an important role in mycoplasma-host interactions (Sachse *et al.*, 1996) have been identified as membrane-associated surface lipoproteins (Vsps) which undergo high-frequency phase and size variation (Rosengarten *et al.*, 1994; Behrens *et al.*, 1994). The present study has defined a new major *M. bovis* antigen, designated pMB67, that has no antigenic or structural similarity to the Vsps, but is endowed with all the attributes characteristic of a functionally important variable antigenic component. Firstly, it is localized on the organism's surface, where it displays a specific mAb-defined epitope. Secondly, it is a prominent integral membrane protein that is, unlike the Vsps, not lipid-modified and apparently does not contain multiple repeating subunits. Thirdly, it undergoes high-frequency phase variation in expression and is size-variable within clonal populations. Moreover, since it can be independently expressed in combination with Vsps, it greatly increases the capacity of this organism to generate and maintain a structurally, antigenically and functionally versatile surface. Finally, it is a dominant target of the bovine immune response during natural infection. Thus, like the Vsps, pMB67 may enable *M. bovis* to exist as a

mixed population with differing properties that are of importance at different stages of infection and may contribute to the ability of this mycoplasma to resist host defences.

The results presented here were highly reproducible and not altered by the passage number of cultures (data not shown). Together with results previously reported (Behrens *et al.*, 1994), they establish that the variable surface antigens of *M. bovis* fall into two distinct classes of integral membrane proteins, namely (i) classical prokaryotic lipoproteins, which are anchored in the membrane by fatty acid moieties covalently linked to an N-terminal cysteine residue in the processed mature product (Wu, 1987; Wise, 1993; Cleavinger *et al.*, 1994), and (ii) nonlipoproteins, which are either membrane-anchored through hydrophobic transmembrane domains in the primary amino acid sequence, as recently shown for the adhesin-like protein PvpA of *Mycoplasma gallisepticum* (Yogev *et al.*, 1994; D. Yogev, D. Menaker & R. Rosengarten, unpublished results), or by other as yet unknown mechanisms, possibly including attachment to lipoprotein by a disulfide bond. While lipoproteins and their genes have now been widely established in mycoplasma surface antigenic variation systems (Rosengarten & Wise, 1990; Yogev *et al.*, 1991; Markham *et al.*, 1993; Wise *et al.*, 1993; Theiss *et al.*, 1993; Behrens *et al.*, 1994; Zheng *et al.*, 1995; Bhugra *et al.*, 1995; Simmons *et al.*, 1996), there are only a few reports defining variable membrane proteins that are not lipid-modified. The only known examples are the P30 adhesin of *M. pneumoniae* (Dallo *et al.*, 1990), the adhesin-like protein PvpA of *M. gallisepticum* (Yogev *et al.*, 1994; D. Yogev, D. Menaker & R. Rosengarten, unpublished results), and several distinct variable surface antigens of *Mycoplasma arthritis* (Droesse *et al.*, 1995). Thus, the data presented here add to the list of variable mycoplasma membrane proteins which are not lipoproteins. Although protein families with structural motifs similar to the *M. bovis* Vsp lipoproteins (Behrens *et al.*, 1994; I. Lysnyansky, R. Rosengarten & D. Yogev, unpublished results) have been found in *Mycoplasma hyorhinis* (Yogev *et al.*, 1991), *Mycoplasma pulmonis* (Bhugra *et al.*, 1995; Simmons *et al.*, 1996) and *Ureaplasma urealyticum* (Zheng *et al.*, 1995), in none of these organisms has the expression of an pMB67-analogous antigen been

demonstrated. Thus, among these mycoplasmas, *M. bovis* is the first reported organism that utilizes for surface diversification two superimposed antigenic variation systems, namely a large repertoire of multiple antigenically related but distinct lipoproteins (Vsps), as well as a single membrane protein (pMB67) which is not lipid-modified. It is also noteworthy that this protein contains cysteine, whilst with one exception (the p43 antigen of *M. arthritis*; Drosse *et al.*, 1995), all other previously described variable surface nonlipoproteins of mycoplasmas do not.

Proteolytic analysis of pMB67 with carboxypeptidase Y revealed a probable surface orientation of this antigen similar to the Vsps (Behrens *et al.*, 1994), with the C-terminal region exposed to the microenvironment surrounding the organism. This suggests that the C-terminal domain is most likely to encounter host defences first and to elicit a dominant antibody response. However, if these antibodies promote mycoplasma killing, only cells expressing the corresponding antigens will be killed. Thus, it is quite possible that variable expression of Vsps and pMB67 may enable *M. bovis* to adhere to cells and invade tissues while ensuring that only a fraction of the population is potentially susceptible to antibody-mediated host defences. However, the precise function of pMB67 and the development and importance of the host immune response to this antigen remains to be elucidated. Detailed analysis of pMB67 structure and variation at the molecular level will be necessary to fully understand its significance in the establishment of systemic infection.

The finding that pMB67 and Vsps (Rosengarten *et al.*, 1994) are selective targets of the humoral immune response in animals infected with *M. bovis* suggests that a cocktail composed of recombinant forms of these antigens might induce an effective prophylactic response to *M. bovis*. In addition, such an antigen cocktail might enable development of a sensitive immunoassay system for specific and rapid identification of *M. bovis*-infected animals. In addition to assessing the use of pMB67 and Vsps as a means of monitoring *M. bovis* infections in the field, future studies should also examine whether antibodies to these antigens are protective to clarify their potential as vaccine antigens.

ACKNOWLEDGEMENTS

The authors would like to thank Vétérinaire Biotechnologie for supplying mAb I₂ and J. L. Martel for many helpful discussions and his generous support.

This work was supported in part by a grant (Ro 739/2-1) from the Deutsche Forschungsgemeinschaft (R. R.) and a grant (LPB-UBA-No. 93-2/2) from the Centre National d'Etudes Vétérinaires et Alimentaires (F. P.).

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Received 7 November 1995; revised 19 April 1996; accepted 9 May 1996.