*Microbiology* (2004), **150**, 4009–4022 **DOI** 10.1099/mic.0.27247-0

# Variability of a glucose phosphotransferase system permease in *Mycoplasma mycoides* subsp. *mycoides* Small Colony

Patrice Gaurivaud,[1] Anja Persson,[2] Dominique Le Grand,[3] Joakim Westberg,[2] Michel Solsona,[1] Karl-Erik Johansson[4] and François Poumarat[1]

Correspondence
François Poumarat
f.poumarat@lyon.afssa.fr

[1]UMR Mycoplasmoses des Ruminants, AFSSA-Site de Lyon, 31 av Tony Garnier, FR-69364 Lyon Cedex 07, France

[2]Department of Biotechnology, Royal Institute of Technology, AlbaNova University Center, SE-106 91 Stockholm, Sweden

[3]UMR Mycoplasmoses des Ruminants, Pathologie du Bétail, Ecole Nationale Vétérinaire de Lyon, 1, Av Bourgelat, FR-69280 Marcy l'Etoile, France

[4]Department of Bacteriology, National Veterinary Institute, SE-751 89 Uppsala and Division of Food Hygiene and Bacteriology DBS-VPH, PO Box 7009, Swedish University of Agricultural Sciences, SE-75007 Uppsala, Sweden

Intraclonal antigenic variation in pathogenic mycoplasma species is considered an important feature of host–pathogen interaction. Such intraclonal protein variation was observed for the interaction of *Mycoplasma mycoides* subsp. *mycoides* Small Colony, the agent of contagious bovine pleuropneumonia, with mAb 3F3. Colony immunostaining allows the definition of 3F3 ON- and 3F3 OFF-type variants, which revert at low frequency. Targets of mAb 3F3 were shown to be surface located, and resided on multiple polypeptides in the 58–68 kDa size range. Phage display and a genomic database were combined to determine the gene encoding the proteins recognized by mAb 3F3. A gene encoding the putative permease of the glucose phosphotransferase system was identified. Genome sequence analysis of strain PG1 revealed two highly similar copies of this gene, resulting from duplication of the chromosomal region carrying the gene. Southern blot analysis demonstrated the presence of this duplication in almost every African strain tested, but not in European strains. DNA analysis revealed that ON/OFF switching is governed by a base substitution occurring upstream of the coding region for the 3F3 epitope. This event generates a stop codon that results in the premature termination of the PtsG protein.

Received 16 April 2004
Revised 3 September 2004
Accepted 7 September 2004

## INTRODUCTION

*Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (*Mmymy* SC) belongs to the class *Mollicutes*, trivial name mycoplasma, whose members are distinguished from other bacteria by their minute size and total lack of a rigid cell wall. *Mmymy* SC is the aetiological agent of contagious bovine pleuropneumonia (CBPP), a highly contagious respiratory disease in cattle. CBPP is the only bacterial disease included in the A list of the World Organization for Animal Health (OIE), which contains prioritized communicable animal diseases. During the nineteenth century, CBPP spread throughout the world, causing extensive losses in livestock. By prohibiting cattle movement, and by a programme of slaughtering and vaccination, CBPP was eradicated during the twentieth century, except in Africa and limited areas of Europe and Asia. Since the late 1980s, CBPP has become the major infectious disease affecting livestock in Africa, and the number of countries with CBPP-infected animals rose dramatically from 15 in the late 1970s to 26 in 2002 [Nicholas *et al.*, 2000; OIE official data (www.oie.int)]. CBPP was thought to be almost eradicated from Europe by the early twentieth century, and until the 1960s only a few sporadic outbreaks continued to occur in the Iberian Peninsula. However, from 1980 onwards, hundreds of outbreaks were reported in France, Portugal, Spain and Italy. Control programmes were set up and no case of CBPP has been officially documented in Europe since 1999 (Portugal).

Abbreviations: CBPP, contagious bovine pleuropneumonia; glucose PTS, phosphoenolpyruvate : glucose phosphotransferase system; HSA, human serum albumin; *Mmymy* SC, *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony.

Although *Mmymy* SC strains are homogeneous (based on protein analysis, Costas *et al.*, 1987), they can be differentiated to some extent at the molecular level. African and European strains can be clearly distinguished by restriction analysis of whole DNA (Poumarat & Solsona, 1995), by the chromosomal distribution of IS*1296* (Cheng *et al.*, 1995; Frey *et al.*, 1995) and by multilocus sequence analysis (Lorenzon *et al.*, 2003). All the *Mmymy* SC strains isolated in Europe since 1980 have undergone a major chromosomal deletion of 8·84 kb, including certain genes in the ABC glycerol transporter operon and a putative lipoprotein *lppB* (Vilei *et al.*, 2000).

In 1990 it was first reported that mycoplasmas can undergo reversible high-frequency variation to alter their surface antigenic repertoire within a cell population (Rosengarten & Wise, 1990). New systems of surface protein variation in pathogenic mycoplasma species are regularly reported, and the observations strongly suggest that such variability confers the means of enhancing colonization and adapting to the host environment during the various stages of infection (Citti & Rosengarten, 1997).

Protein variation not only enables mycoplasmas to escape the host immune defence system (Citti *et al.*, 1997; Neyrolles *et al.*, 1999; Le Grand *et al.*, 1996), but is also involved in adhesion (Sachse *et al.*, 2000; Washburn *et al.*, 1993), haemadsorption (Markham *et al.*, 1993; Noormohammadi *et al.*, 1997, 2000), membrane transport (Theiss & Wise, 1997) and immunomodulation (Muhlradt *et al.*, 1998). The simplest form of surface variation is a reversible ON/OFF switch in protein expression, called phase variation. The wide range of mutations involved in such variation include promoter mutations (Yogev *et al.*, 1991; Persson *et al.*, 2002) or inversion (Horino *et al.*, 2003), changes in putative transcription activators (Glew *et al.*, 1998; Washburn *et al.*, 1998), mutations that lead to gene truncation (Theiss & Wise, 1997), frameshift mutations (Boguslavsky *et al.*, 2000; Winner *et al.*, 2003; Zhang & Wise, 1997) and DNA inversions or gene conversions that fuse the coding gene to an active promoter (Bhugra *et al.*, 1995; Glew *et al.*, 2002; Noormohammadi *et al.*, 2000; Lysnyansky *et al.*, 2001b).

Some surface proteins undergo antigenic variation by altering the size and/or epitope composition. Most of these proteins contain repetitive units of different structure. The repetitive sequences function like interchangeable cassettes that can be deleted, inserted or recombined to form a rich variety of protein variants (Zheng *et al.*, 1995; Zhang & Wise, 1996; Yogev *et al.*, 1995; Lysnyansky *et al.*, 1999, 2001a; Boguslavsky *et al.*, 2000; Boesen *et al.*, 1998; Citti *et al.*, 2000). Epitope masking is a phenomenon in which the epitopes of a constitutively expressed surface protein are subject to variable surface exposure, either due to a secondary protein that sterically blocks accessibility of the surface epitopes or as a consequence of size variation (Citti *et al.*, 1997; Zhang & Wise, 2001). Variable proteins are often subjected to both size and phase variation, and many are members of multigene families. The proteins of a multigene family are often differentially expressed, and chromosomal rearrangements occur to allow transcription of one gene instead of another (Lysnyansky *et al.*, 1999; Citti *et al.*, 2000; Markham *et al.*, 1994, 1999; FlitmanTene *et al.*, 2000; Glew *et al.*, 2000; Roske *et al.*, 2001; Shen *et al.*, 2000; Horino *et al.*, 2003; Noormohammadi *et al.*, 1998).

*Mmymy* SC was originally thought not to exhibit intraclonal variability, but a variable surface protein Vmm (Persson *et al.*, 2002) that undergoes reversible phase variation has recently been identified. The *vmm* gene has been demonstrated in all *Mmymy* SC strains as a single copy and is regulated at the transcriptional level by dinucleotide (AT) insertions or deletions in a repetitive region of the promoter spacer. Genome sequencing has shown that five additional genes encoding prolipoproteins that have promoters with variable TA-repeats are located upstream of the *vmm* gene and may belong to the same family (Westberg *et al.*, 2004).

Studies of the epitope recognized by mAb 3F3 started because the epitope is specific to *Mmymy* SC and because it is recognized in the host immune response (Brocchi *et al.*, 1993). These features make 3F3 useful for diagnostic analysis. Unexpectedly, antigenic variation was observed with this mAb, indicating that *Mmymy* SC is undergoing a new phase variation.

## METHODS

**Mycoplasma strains and isolates.** Strains were propagated in liquid medium as previously described (Poumarat *et al.*, 1991). All mycoplasma strains used in this study and their origins are listed in Table 1. They were chosen to be from different origins (Africa, Australia and Europe) and to be representative of the different genomic profiles obtained by Cheng *et al.* (1995). A low-passage isolate of *Mmymy* SC, strain M 223/90, obtained from a bovine that had died of severe CBPP, was used to make a phagemid library. Four strains were used to investigate clonal variability and to prepare subclones of phenotypic variants: i) the highly virulent (Abdo *et al.*, 1998; Belli *et al.*, 1989) *Mmymy* SC strain Afadé, fourth-passage isolate from a cow that had died from acute CBPP after experimental infection (Belli *et al.*, 1989); ii) the low-virulence *Mmymy* SC strain T1/44/2, which is the only strain recommended by the World Organization for Animal Health for preparing live vaccines – direct culture from a vaccine vial (batch EMVT002 PANVAC MVT) was used in this study; iii) the *Mmymy SC* strain B17, the virulence of which is still unknown; iv) the avirulent *Mmymy* SC strain KH3J, formerly used as a live vaccine in Africa (Tulasne *et al.*, 1996).

Direct lineages of subcloned variants were prepared as follows. Fresh broth cultures of organisms from the strain stocks were passed through a 0·45 μm pore-size filter and then serially diluted and plated on agar. After 4 days incubation, single colonies that were positive for mAb 3F3 were located by aligning them with corresponding coloured dots on a nitrocellulose membrane screened by colony immunostaining (see below). The colonies were picked with Pasteur pipettes and propagated at 37 °C in 1 ml broth medium for 4 days. After three successive steps of subcloning, the resulting broth cultures were stored at −80 °C for further characterization by Western blot analysis, except for 100 μl that was replated as above. After 4 days incubation, the few reverting negative colonies were located by aligning them with corresponding ponceau-stained dots on colony blots, and subsequently purified by several rounds of subcloning as above. Negative colonies of the third

**Table 1.** *Mmymy* SC strains used in this study

Abbreviations and addresses: CIRAD-EMVT, Centre de Coopération Internationale en Recherche Agronomique Pour le Développement, Département d'Elevage et de Médecine Vétérinaire des pays Tropicaux, Santé Animale, FR-34398, Montpellier cedex 5, France; SVA, National Veterinary Institute, Department of Bacteriology, SE-751 89 Uppsala, Sweden; AFSSA, Agence Française de Sécurité Sanitaire des Aliments – site de Lyon 31 av. Tony Garnier, FR-69364, Lyon cedex 07; LNV, Laboratorio National de Investigaçao Veterinaria, Estrada de Benfica 701, PT-1500 Lisboa, Portugal; NCTC, National Collection of Type Cultures, PHLS, London, UK; SIMA, SIMA Centro Derio Berroaga Kalea 1, ES-48760 Derio.

IS*1296* patterns were established as described by *Cheng et al.* (1995), using the same nomenclature. ND, Not done. *ptsG* gene profiles were obtained by Southern blot with the *ptsG* PCR probe. 1 band, One band of 4000 bp; 1 atypical band, one band of 2000 bp; 2 bands, one band of 4000 bp and one band of 7800 bp.

| Strain | Collection | Continent | Country | Date isolated | Host | IS*1296* profile | *ptsG* gene profile |
|---|---|---|---|---|---|---|---|
| M375 | SVA | Africa | Botswana | 1995 | Cattle | Atypical A | 2 bands |
| B17 | CIRAD EMVT | Africa | Chad | 1967 | Zebu | A1 | 2 bands |
| 9381 | AFSSA | Africa | Mauritania | 1995 | Cattle | A1 | 2 bands |
| Fatick | CIRAD EMVT | Africa | Senegal | 1968 | Cattle | A1 | 2 bands |
| 2162 | CIRAD EMVT | Africa | Senegal | pre-1990 | Cattle | A1 | 2 bands |
| Filfili | CIRAD EMVT | Africa | Senegal | pre-1989 | Cattle | A3 | 2 bands |
| KH3J | CIRAD EMVT | Africa | Sudan | 1940 | Cattle | A2 | 2 bands |
| MX696 | SVA | Africa | Tanzania | 1999 | Goat | A1 | 2 bands |
| T1/44/2 | CIRAD EMVT | Africa | Tanzania | 1952 | Cattle | A4 | 2 bands |
| MK102 | SVA | Africa | Tanzania | 1999 | Cattle | A4 | 2 bands |
| M223/90 | SVA | Africa | Tanzania | 1990 | Cattle | ND | 2 bands |
| Gladysdale | SVA | Australia | Australia | pre-1964 | Cattle | A1 | 2 bands |
| PG1 | NCTC | Unknown | Unknown | 1931 | Cattle | P | 2 bands |
| Afadé | CIRAD EMVT | Africa | Chad | 1968 | Cattle | A1 | 2 bands |
| Gemu Gofa | CIRAD EMVT | Africa | Ethiopia | 1974 | Cattle | A1 | 2 bands |
| Dakar 2144 | CIRAD EMVT | Africa | Senegal | pre-1988 | Cattle | A1 | 1 band |
| V5 | SVA | Australia | Australia | 1936 | Cattle | A7 | 1 band |
| PO-67 | CIRAD EMVT | Europe | France | 1967 | Cattle | A1 | 1 band |
| 2022 | AFSSA | Europe | France | 1984 | Cattle | E1 | 1 band |
| 2091 | AFSSA | Europe | France | 1984 | Cattle | E1 | 1 band |
| 2092 | AFSSA | Europe | France | 1984 | Cattle | E1 | 1 band |
| 2117 | AFSSA | Europe | France | 1984 | Cattle | E1 | 1 band |
| 4813 | AFSSA | Europe | Italy | 1990 | Cattle | E1 | 1 band |
| 6466 | AFSSA | Europe | Italy | 1992 | Cattle | E1 | 1 band |
| 6671 | AFSSA | Europe | Italy | 1993 | Cattle | E1 | 1 band |
| 6865 | AFSSA | Europe | Italy | 1993 | Cattle | E1 | 1 band |
| 6866 | AFSSA | Europe | Italy | 1993 | Cattle | E1 | 1 band |
| 6970 | AFSSA | Europe | Italy | 1992 | Cattle | E1 | 1 band |
| 6981 | AFSSA | Europe | Italy | 1992 | Cattle | E1 | 1 band |
| 6479 | AFSSA | Europe | Italy | 1992 | Cattle | E2 | 1 band |
| B103 | LNV | Europe | Portugal | 1986 | Cattle | E1 | 1 band |
| C 425 goat | LNV | Europe | Portugal | 1993 | Goat | E1 | 1 band |
| O 512 sheep | LNV | Europe | Portugal | 1993 | Sheep | E1 | 1 band |
| B 345 | LNV | Europe | Portugal | ? | Cattle | E1 | 1 band |
| 2059 | AFSSA | Europe | Spain | 1984 | Cattle | E1 | 1 band |
| 6364 | AFSSA | Europe | Spain | 1991 | Cattle | E1 | 1 band |
| Madrid | SVA | Europe | Spain | ? | Cattle | E1 | 1 band |
| Segovia | SVA | Europe | Spain | ? | Cattle | E1 | 1 band |
| Asturia | SVA | Europe | Spain | ? | Cattle | E1 | 1 band |
| Santander | SVA | Europe | Spain | ? | Cattle | ND | 1 band |
| 7170 | AFSSA | Europe | Italy | 1993 | Cattle | Atypical E | 1 atypical band |
| 4878 | AFSSA | Europe | Italy | 1990-94 | Cattle | E1 | 1 atypical band |
| 6363 | AFSSA | Europe | Spain | 1991 | Cattle | E1 | 1 atypical band |

generation of 3F3-negative subclones were propagated in 1 ml broth and were stored at −80 °C, except for 100 µl that was replated. Reverting 3F3-positive subclones were prepared as above. Oscillating phenotypic switches were quantitatively monitored and expressed as the fraction of switched phenotype per cell per generation.

**mAb, oligopeptide and immunoassays.** mAb 3F3 was prepared from *Mmymy* SC type strain PG1 in collaboration with the AFSSA-Site de Lyon and the Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia (Brocchi *et al.*, 1993). Dot blotting was performed as described by Poumarat *et al.* (1991). Colony immunostaining was carried out as described by Rosengarten & Yogev (1996). Freshly grown mycoplasma colonies were transferred to nitrocellulose membranes by placing the membranes on the surface of agar plates. The membranes were gently removed and blocked in TBS-B buffer [TBS (50 mM Tris and 0·2 M NaCl) supplemented with 10 % horse serum], before they were incubated with mAb 3F3 (at a concentration of 9 µg $ml^{-1}$) diluted at 1/1000 in TBS-B, for 1·5 h at ambient temperature. Unbound antibody was removed by washing three times in TBS-T (TBS and 0·05 % Tween 20) and once in TBS. Thereafter, the membranes were incubated with peroxidase-conjugated rabbit anti-mouse antibodies (DAKO) in TBS-B (2·6 µg $ml^{-1}$) for 1·5 h, followed by three washes in TBS-T and one wash in TBS. Colonies expressing proteins that bind mAb 3F3 were specifically identified by an enzymic colour reaction with 4-chloro-1-naphthol, which gives a dark blue colour. The membranes were finally stained with ponceau red solution (Sigma Diagnostics), which non-specifically stains proteins red, to reveal colonies negative for mAb 3F3.

Western blotting was carried out as previously described (Le Grand *et al.*, 1996) after SDS-PAGE in 8–16 % gradient polyacrylamide gels.

The synthetic peptide containing the target epitope for mAb 3F3, which covered the region that was shared by all phagemid clones, was obtained from Interactiva Biotechnologie. The peptide sequence was acetyl–NH–NTQSEEVKKAFVDSYNKLHGTNHNNLKAI–$CONH_2$.

**Immunoelectron microscopy.** The ON-type and OFF-type variants of the Afadé strain were subjected to immunoelectron microscopy. Colonies of *Mmymy* SC were grown on agar plates and resuspended in 200 µl 0·05 M Tris/HCl buffer (pH 7·4) with 1 % BSA. One drop of cell suspension was placed on a 200 mesh nickel grid and coated with 1/1000 poly-L-lysine for 5 min, before being blocked for 5 min with Tris/HCl buffer (pH 7·4) containing 1 % BSA. Grids were transferred to a 50 µl drop of mAb 3F3 diluted 1 : 100 in Tris/HCl buffer (pH 7·4) with 1 % BSA and incubated for 15 min at room temperature. After rinsing twice for 5 min in Tris/HCl buffer (pH 7·4) and once for 5 min in Tris/HCl buffer (pH 8·2), the grids were pre-incubated for 5 min in Tris/HCl buffer (pH 8·2) with 1 % BSA. Grids were then incubated for 15 min at room temperature with the secondary antibody: gold conjugate anti-mouse immunoglobulins (10 nm) (Tebu, France) diluted 1 : 40 in Tris/HCl buffer (pH 8·2) with 1 % BSA. After rinsing twice for 5 min in Tris/HCl buffer (pH 8·2), once for 5 min in Tris/HCl buffer (pH 7·4) and once for 5 min in filtrated distilled water, the grids were negatively stained with 0·3 % (w/v) phosphotungstic acid (pH 7·0), blotted dry and observed with a JEOL 1200 EX transmission electron microscope equipped with numeric Megaview II camera and AnalySIS software.

**Phage display.** A gene VIII-based whole-genome phage-display library of randomly fragmented chromosomal DNA from strain M223/90 of *Mmymy* SC (Persson *et al.*, 2002) was used to identify the target epitope of mAb 3F3. The library was made from a mixture of phagemid vectors pG8PL0 and pG8SPA0 (Jacobsson & Frykberg, 1998). It contained $10^7$ independent clones and the titre of the phage stock was $10^{11}$ p.f.u. Both vectors were constructed so that the mycoplasma peptides were fused to a tag that binds human serum albumin (HSA), and permitted ligand-independent screening of clones that expressed mycoplasma peptides.

Affinity selection of phage that displayed mycoplasma peptides recognized by mAb 3F3 was then carried out by three consecutive pannings. Two Maxisorp microtitre wells (Nalge Nunc International) were coated with 125 µg protein G in 250 µl coating buffer (0·05 M $Na_2CO_3$, pH 9·7) for 1 h at room temperature. The wells were rinsed three times with PBS containing 0·05 % Tween 20, before addition of 250 µl mAb 3F3 diluted 1/3000 in PBS in one of the wells and BALB/c mouse serum in the other well as a negative control. The antibodies were immobilized for at least 1 h and the wells were then rinsed six times with PBS/Tween and blocked with PBS/Tween for 10 min. Meanwhile, the HSA-binding region of the phagemids was blocked by incubating the phage stock with 100 µg HSA $ml^{-1}$. After 1 h, 200 µl phage stock was transferred to each coated well, and the panning proceeded for 4 h at room temperature. The wells were rinsed 30 times in PBS/Tween, to remove unspecifically bound phage, then the captured phage was eluted with 200 µl sodium citrate buffer (50 mM sodium citrate, 140 mM NaCl, pH 2). The eluates were neutralized with 40 µl 2 M Tris/HCl buffer (pH 8·7), serially diluted in Luria–Bertani (LB) medium and immediately used to infect a 150 µl overnight culture of *E. coli* CDJ64/Δ14 (Rydén & Isaksson, 1984). The infected cultures were incubated for 30 min at room temperature, spread on selective Luria agar (LA) plate (Sigma) and incubated overnight. The colonies were then counted, and 150 were transferred to a new selective LA plate for colony blot screening. New phage stocks were produced by resuspending the remaining colonies from the 3F3 panning in LB medium, infecting the bacteria with helper phage R408 and processing as above. A conjugate of HSA and horseradish peroxidase was used in the colony blot screening to detect the tag. Replica blots were screened with mAb 3F3 to identify the clones that expressed the 3F3 epitope.

**DNA sequencing.** The mycoplasma inserts from phagemid clones that were positive for the tag and for mAb 3F3 by colony blot screening were sequenced. Phagemids were prepared with the Wizard Plus SV Miniprep DNA purification system (Promega), and the inserts first sequenced with the ALBP primer (Table 2), which is complementary to the HSA-binding region of the vector. Depending on the mycoplasmal DNA vector insertion, the phagemids were then sequenced with the Sasekv or Nypel primers (Table 2). The complete sequence of the *ptsG* gene and flanking region was determined after searching the genome database of *Mmymy* SC strain PG1 with the consensus sequence (Westberg *et al.*, 2004).

In order to investigate the genetic mechanism involved in 3F3 ON/OFF switching, chromosomal DNA was extracted from i) liquid culture prepared from the 3F3-ON variant of the Afadé strain and showing only 3F3-ON colonies after plating on solid medium and colony immunostaining and ii) liquid culture prepared from a 3F3-OFF variant from the Afadé strain, originating from the same lineage as the 3F3-ON variant above and showing only 3F3-OFF colonies after plating on solid medium and colony immunostaining. The 1·3 kpb DNA sequence from the end of IS*1634*AL to the region downstream of the sequence encoding the 3F3 epitope was amplified by PCR using primers IS*1634*For and PGRev (Table 2) and sequenced with primers IS*1634*For, PGFor, PGI1, PGI2 and PGrev. All DNA sequencing in this study was performed with the ALFexpress DNA Sequencer and the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP or the Thermo Sequenase fluorescent dye terminator kit (Amersham Pharmacia Biotech), according to the manufacturer's description. DNA sequences were assembled with ASSEMGEL, a software in the PC/Gene package (IntelliGenetics Inc.).

**Southern blot hybridization.** The presence of the *ptsG* gene in 43 *Mmymy* SC strains (Table 1) was assessed by Southern blotting as

**Table 2.** Oligonucleotides used for DNA sequencing, PCR and Southern blotting

| Primer | Orientation | Sequence | 5′ Label |
|---|---|---|---|
| ALBP | Reverse | 5′-GCCATACTGCTTTAGTTCATTGAT-3′ | Cy5 |
| Sasekv | Forward | 5′-TATCTGGTGGCGTAACACCTGCT-3′ | Cy5 |
| Nypel | Forward | 5′-CCTATTGCCTACGGCAGCCGCTGG-3′ | Cy5 |
| 3F3-forward | Forward | 5′-TACTTACTAGTATTAACTATTGGA-3′ | |
| 3F3-reverse | Reverse | 5′-TTTAGTATATGCTAAAGCTACAG-3′ | |
| 3F3-insert probe | Forward | 5′-AGGCATTCGTTGATTCATATA-3′ | DIG |
| IS*1634*For | Forward | 5′-CTCCCACAAAATCGACAACA-3′ | |
| PGFor | Forward | 5′-GATATACTATTATAAAGAGG-3′ | |
| PGI1 | Forward | 5′-GGAGTTGCAGCAATTACTGC-3′ | |
| PGI2 | Reverse | 5′-CAGGAGCAATAAATAAAGG-3′ | |
| PGRev | Reverse | 5′-GTGACCAATAACTCTTTGCG-3′ | |

described by Poumarat *et al.* (1999). Chromosomal DNA was digested to completion with the restriction enzyme *Hin*dIII and the fragments were separated by electrophoresis on 0·8 % agarose gel. Two probes were used: i) the 3F3-insert probe (Table 2), corresponding to an inner part of the 3F3 epitope sequence (hybridization at 44 °C), and ii) the *ptsG* PCR probe (hybridization at 65 °C). The latter probe was produced by PCR with the 3F3-forward and 3F3-reverse primers (Table 2) with DNA from strain PG1 as template. The reaction mixtures were prepared with the PCR DIG Probe Synthesis Kit (Roche Diagnostic).

**Nucleotide sequence accession number and sequence analysis.** The nucleotide sequences for the two copies of the *ptsG* gene were assigned the ORF names MSC_0860 and MSC_0873 in the genome sequence database of the *Mmymy* SC strain PG1 (accession no. NC_005364). The ProDom database was used to study the protein domain arrangements (Corpet *et al.*, 1999). Multiple sequence alignment was performed with the Multalin program (Corpet, 1988).

## RESULTS

### mAb 3F3-binding proteins are surface-located and undergo phase variation

All the 43 *Mmymy* SC strains tested by dot immunobinding (Table 1) were positive to mAb 3F3, except strain KH3J. Staining level varied, the T1/44/2 and B17 strains reacting particularly weakly. These latter strains and the strongly positive Afadé strain were further investigated by colony immunostaining with mAb 3F3 directly on first-passage culture from collection vials before any cloning. All three strains proved to be mixtures of positive, negative and mixed colonies, but the proportions varied considerably: 99·9 % positive colonies for the fourth *in vitro* passage isolate of Afadé, 60 % for B17, 50 % for strain T1/44/2 and less than 0·001 % for strain KH3J (only weakly sectored colonies). Single positive and negative colonies were cloned three times for each strain and cell generations from these subclones were then analysed by colony immunostaining. Fig. 1(a) shows colony immunostaining with mAb 3F3 of a subclone of the Afadé strain: the mixtures of positive, negative and sectored colonies, in particular, indicate that the target protein which binds mAb 3F3 undergoes intraclonal phenotypic variation. Similar pictures were obtained with a subclone of T1/44/2 (Fig. 1b) and with B17 strains (not shown). An attempt to clone an ON variant of the KH3J strain was not successful. The ON/OFF switching rate varied greatly according to strains and subclones. For strains B17 and Afadé, the reversion from ON to OFF type ranged from $10^{-4}$ to less than $10^{-6}$ per cell per generation, and reversion from OFF to ON occurred at frequencies ranging from $10^{-2}$ to less than $10^{-6}$. The ON/OFF switching rate for the vaccine strain T1/44/2 never exceeded $10^{-6}$.

Immunoblotting of whole-cell proteins from ON and OFF variants of direct clonal lineages of Afadé, T1/44/2 and B17 strains (Fig. 2) gave three or four bands ranging from 58 to 68 kDa in ON-type variants, whereas no protein band was detected in OFF-type variants. This proved that the phenotypic variation observed in the immunostained colony resulted from reversible phase variation in expression of the proteins binding mAb 3F3, and was not due to epitope masking or size variation.

Immunoelectron microscopy of the ON type and the OFF type (data not shown) of the Afadé strain showed that mAb 3F3 target proteins were expressed at the surface of the mycoplasmal membrane in the ON type (but not in the OFF type).

### Identification and putative function of the gene encoding the protein that binds mAb 3F3

Serial affinity pannings of the phage-display library to mAb 3F3 resulted in an accumulation of phages that expressed peptides containing the target epitope of this mAb. The total amount of recovered phage determined from the viable count of infected *E. coli* was $2{\cdot}5 \times 10^4$, $4{\cdot}0 \times 10^4$ and $2{\cdot}2 \times 10^6$ c.f.u. of the first, second and third pannings, respectively. Colony blot screening for expression of the tag in 150 of these colonies gave 5, 46 and 140 HSA-positive colonies. In the original phage stock, two colonies out of 150 were positive for HSA. Replica blots which were screened with mAb 3F3 showed that nearly all the tag-positive colonies were also positive for mAb 3F3. Control pannings with BALB/c serum performed in parallel with the mAb 3F3 pannings resulted in considerably lower

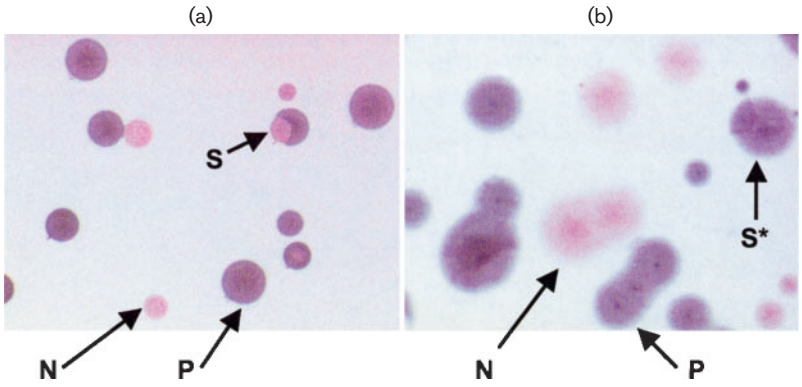


**Fig. 1.** Colony immunostaining with mAb 3F3 of *Mmymy* SC strains showing variable phenotypical expression of the PtsG proteins at the surface among and within colonies. The colonies are derived from broth cultures which were filtered through a 0·45 μm pore-size filter and plated on agar. (a) Subclone of *Mmymy* SC strain Afadé showing positive, negative and sectored colonies. (b) Direct culture from a vaccine vial of *Mmymy* SC strain T1/44/2. The population consists of: positive colonies (P) for mAb 3F3 that express PtsG proteins; negative colonies (N) (unspecifically stained by ponceau red); sectored colonies (S) in which mutation during growth has induced positive to negative expression switching of the PtsG proteins; sectored colonies (S*) in which mutation during growth has induced weaker to stronger staining of the PtsG proteins.

amounts of recovered phagemids, namely $1{\cdot}3 \times 10^4$, $5{\cdot}9 \times 10^3$ and $6{\cdot}0 \times 10^2$ c.f.u., respectively. Twenty colonies that were positive for both the tag and for mAb 3F3 were randomly selected from the third panning. The phagemid DNA was then isolated and the mycoplasmal inserts sequenced. An overlapping sequence covering a particular region was detected in all inserts, thus limiting the 3F3 epitope to an 85 bp gene region that was present in all clones. Several colonies turned out to be multiples of the same recombinant, but there were eight unique recombinants in which the gene segments differed in size. When the panning experiments were repeated, another five clones from the second panning were sequenced. The mycoplasmal inserts of these clones also contained the 85 bp gene region. The panning results were further evaluated by producing a synthetic peptide of 29 amino acids corresponding to the 85 bp region. Dot blotting of the synthetic peptide and detection with mAb 3F3 resulted in strong positive signals (data not shown). When the consensus of the mycoplasma inserts was compared to the genome database of *Mmymy* SC strain PG1 (Westberg *et al.*, 2004), sequence data were retrieved for the full gene and its flanking regions. Two genes (MSC_0860 and MSC_0873) were found, both annotated *ptsG*. Using the BLAST program (Altschul *et al.*, 1997), we showed that MSC_0860 and MSC_0873 are identical, except in position 113, where the GCT (Ala)

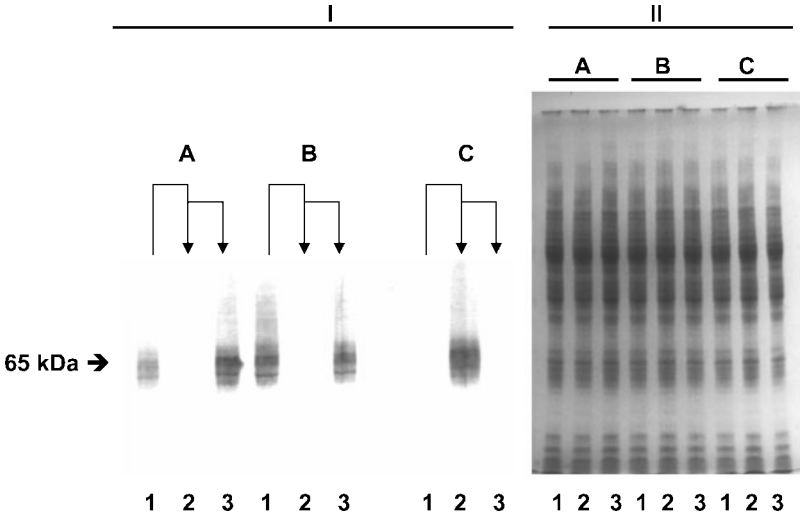


**Fig. 2.** Whole-cell proteins of serially subcloned phenotypic variants selected by colony immunostaining from three *Mmymy* SC strains that were separated in a 8–16 % polyacrylamide gel and immunoblotted with mAb 3F3, which targets PtsG proteins. Sequential phenotypic (ON/OFF) transitions of PtsG protein expression are indicated with arrows to represent direct lineages of the subcloned variants. Panels A, B and C show samples from *Mmymy* SC strains Afadé, T1/44/2 and B17, respectively. In panels A and B, lanes 1 were ON-type subclones, lanes 2 were OFF-type revertant subclones and lanes 3 were ON-type double-revertant subclones. In panel C, lane 1 was OFF-type subclone, lane 2 was ON-type revertant subclone and lane 3 was OFF-type double-revertant subclone. An equal quantity of protein was loaded in each well, as determined by SDS-PAGE of samples stained with Coomassie Brilliant Blue (shown in panel II).

codon in MSC_0860 is replaced by the GTT (Val) codon in MSC_0873. Translation could theoretically occur from seven putative start codons, two of which are predicted to produce a signal sequence (Nielsen *et al.*, 1997). The first signal sequence, which starts at the first or second methionine, was predicted to be cleaved off between amino acid positions 49 and 50 in AIA-AN residues. A putative ribosome-binding site (AAAGAA in MSC_0860 and AAAGGA in MSC_0873) is located 12 bp upstream of the first start codon. Promoter regions TATTAA and TATAAT were identified 108–80 bp before the same start codon. The longest ORF (2028 bp) would encode a native protein of 676 aa (about 75 kDa).

The *ptsG* gene encodes the permease of the phosphoenolpyruvate : glucose phosphotransferase system (glucose PTS). Bacterial sugar phosphotransferase catalyses the concomitant transport and phosphorylation of its sugar substrate.

*Mmymy* SC PG1 genome analysis (Westberg *et al.*, 2004) revealed two other ORFs annotated *ptsG*, designated MSC_0054 and MSC_0161, but they do not harbour the protein sequence corresponding to the epitope recognized by mAb 3F3.

The protein sequence deduced from MSC_0860 and MSC_0873 showed 69 % similarity with PtsG of *Spiroplasma citri* (accession no. AAP55652), 56 % similarity with PtsG of *Mycoplasma pulmonis* (accession no. NP_325848) and 52 % similarity with PTS of *Mycoplasma penetrans* (accession no. NP_757560.1).

The protein sequence obtained from MSC_0054 shows 48 % similarity with PtsG of *S. citri* (accession no. AAP55652), 51 % similarity with PtsG of *M. pulmonis* (accession no. NP_325848), 64 % similarity with PtsG of *M. penetrans* (accession no. NP_757560.1) and 47 % similarity with MSC_0860 and MSC_0873.

MSC_0161 encodes a putative protein showing homology only with the N-terminal part of PtsG and which could be described as truncated. However, nucleic acid analysis showed that a complete PtsG protein could be obtained by removing three frameshifts (data not shown).

## PtsG protein structures

PTS permeases consist of several distinct or fused polypeptide chains (Saier & Reizer, 1992). MSC_0860 and MSC_0873 code for a native protein of 676 aa (about 75 kDa) and MSC_0054 for a protein of 580 aa (about 65 kDa). Protein sequence analysis, deduced from MSC_0054, MSC_0860 and MSC_0873 by using ProDom (Corpet *et al.*, 1999), indicates that these proteins consist of three domains linked to each other as follows: IIC domain, unidentified domain and IIB domain. Although the sequence signature of the IIB domain was found in all PtsG of *Mmymy* SC, it is not the consensus sequence described for most bacteria (i.e. N-[LIVMFY]-x(5)-C-x-T-R-[LIVMF]-x-[LIVMF]-x-[LIVM]-x-[DQ]; Reizer *et al.*, 1994). In MSC_0054, [DQ] is replaced by N and in MSC_0860 and MSC_0873, T is substituted by S. These substitutions are found in other bacteria, such as *M. pulmonis* and *Pasteurella multocida*. Immediately downstream of the IIC domain, a short sequence was observed containing two histidyl residues. It showed a low similarity to the sequence signature of the IIA domain and could be a degenerated IIA domain. However, a consensus sequence signature of the IIA domain was not found in the PtsG of *Mmymy* SC. Moreover, a multiple sequence alignment between the PtsG proteins of *Mmymy* SC and those of other mycoplasmas clearly confirmed the absence of a IIA domain in *Mmymy* SC. The observed structure of *Mmymy* SC differed from the IICBA organization described for the PTS mycoplasmas. However, an absence of IIA–IICB fusion has also been observed for the phytopathogenic mollicute *S. citri* (André *et al.*, 2003).

The IIC domain (amino acids 42–445 according to ProDom) deduced from MSC_0860 and MSC_0873 shows eight transmembrane regions as predicted by TopPred2 (Claros & von Heijne, 1994). The 85 bp gene region corresponding to the epitope recognized by mAb 3F3 is located between two conserved segments of the IIC domain at amino acid position 271–299. This part of the gene is predicted to be in a loop of 87 aa at the outside of the cell surface between membrane helices 4 and 5. The sequence of the mAb 3F3 epitope did not show any similarity with any other genes or peptides in the NCBI database.

## The *ptsG* gene in the *Mmymy* SC genome

MSC_0860 and MSC_0873 occur in a long chromosomal DNA direct repeat (Fig. 3). The observed organization of the regions containing MSC_0860 and MSC_0873 is similar. Differences are due to the insertion and orientation of IS*1634* as well as to mutations creating frameshifts in the ORFs. Each repeat contains an IS*1634* copy (IS*1634*AX and IS*1634*AZ according to Westberg *et al.*, 2004) inserted in different sequences. IS*1634*AX is inserted 86 bp after the start codon of the *glk* gene and, in consequence, *glk* is truncated at its 5′ extremity. In the MSC_0873 region, the *glk* gene does not seem to be functional because of a frameshift resulting from the insertion of two guanosines 201 bp after the start codon of the *glk* gene. This gene encodes a putative protein with the sequence signature of the ROK (Repressor, ORF, Kinase) family. This family regroups transcriptional repressors, sugar kinases and uncharacterized ORFs (Titgemeyer *et al.*, 1994). Repressor proteins in this family possess an N-terminal region which contains an helix–turn–helix DNA-binding motif. No such motif could be identified in the protein deduced from the *glk* gene, even when the frameshift was removed to restore the complete gene. This suggests that the protein in question is a sugar kinase. However, because of the frameshift or the IS*1634* insertion, neither *glk* gene should be functional.

IS*1634* is characterized by the presence of direct repeats

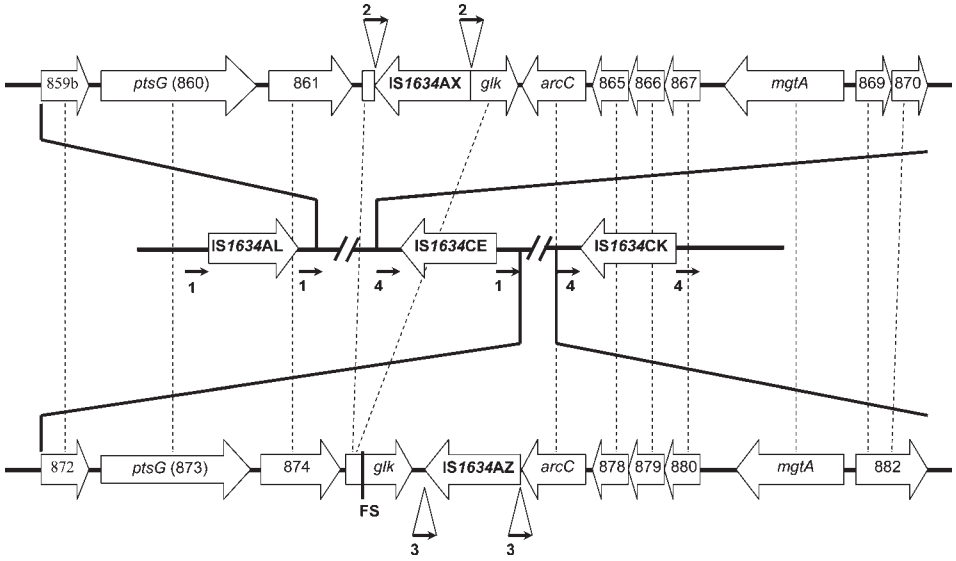


**Fig. 3.** Schematic representation of the chromosomal region carrying the *ptsG* genes MSC_0860 and MSC_0873. Numbers in boxes correspond to the ORFs annotated by Westberg *et al.* (2004). Direct repeats bordering the IS*1634* copies are indicated by arrows and the type number. FS, frameshift in the glucokinase gene (*glk*); *mgtA*, gene encoding a hypothetical magnesium transport protein; *arcC*, gene encoding a hypothetical carbamate kinase. Homologous genes are indicated with broken lines.

which vary in length at the site of insertion (Vilei *et al.*, 1999). Direct repeats were identified by comparison of IS*1634* (accession no. AF062493) with sequences overlapping the extremities of insertion sequences present in the region analysed. The IS*1634* copies present inside the chromosomal repeats are bordered by different direct repeats: a 9 bp repeat (5′-ATAGTTTTC-3′) is observed for IS*1634*AX present in the MSC_0860 region and a 10 bp repeat (5′-AGCTTGTTCT-3′) for IS*1634*AZ in the MSC_0873 region (Fig. 3). The chromosomal duplication is framed by IS*1634*AL and IS*1634*CK. The direct repeat for IS*1634*AL is a 19 bp sequence (5′-AATTAAACTTCT-TTTTTTT-3′), and a 13 bp sequence (5′-AGGTTCTAT-ATTT-3′) was found for IS*1634*CK. The situation in IS*1634*CE, separating the two chromosomal repeats, is not so clear: no direct repeat framing of this insertion sequence could be identified, although the direct repeat of IS*1634*CK was detected at the 5′ end of IS*1634*CE and the direct repeat of IS*1634*AL was found at the 3′ end of IS*1634*CE (Fig. 3).

## Occurrence of the *ptsG* gene in field isolates of *Mmymy* SC

Forty-three *Mmymy* SC strains were subjected to Southern blot on *Hin*dIII-digested genomic DNA and hybridization with the *ptsG* PCR probe (Table 1). Both the *ptsG*-PCR probe and the specific 3F3 insert probe were tested beforehand on 10 strains, and both hybridizations gave the same profiles (Fig. 4). Although all *Mmymy* SC strains contained the *ptsG* gene, three distinct patterns were obtained (Fig. 4 and Table 1). All 26 strains isolated in Europe showed only a single band which was 4 kb in size in 23 strains and 2 kb in size in three strains (two from Italy and one from Spain). All strains from Africa, like the PG1 strain, had the same profile with two bands (7·8 kb and 4 kb) except one from Senegal, which showed a European profile. The profile of the Australian Gladysdale strain was of African type, whereas that of the V5 Australian vaccine strain was of European type. The two *ptsG* genes of the PG1 strain detected by the probes showed only three *Hin*dIII restriction sites that were in the 3′ distal part of the gene outside the target sequences of the two probes. Thus, the *Hin*dIII fragments of *Mmymy* SC could not contain more than one copy of the *ptsG* gene, which could explain why European strains with a single band profile harbour only one copy of the *ptsG* gene. The *Hin*dIII restriction pattern, deduced from the DNA sequence of the MSC_0860 and MSC_0873 regions, indicates that the 4 kb fragment revealed by Southern blot hybridization corresponds to the MSC_0860 region, and that the 7·8 kb fragment corresponds to the MSC_0873 region.

## Mechanism of PtsG protein phase variation

As we had demonstrated that MSC_0860 is present in all *Mmymy* SC strains tested, we chose to amplify and sequence this genomic region from 3F3-ON and 3F3-OFF variants obtained from the same lineage of the Afadé strain. The GCT (Ala) codon confirmed the amplification of MSC_0860 instead of MSC_0873. Comparison of the 1·3 kb sequence from the end of IS*1634*AL to the region downstream of the sequence encoding the 3F3 epitope revealed a nucleotide change (G to A) only at position

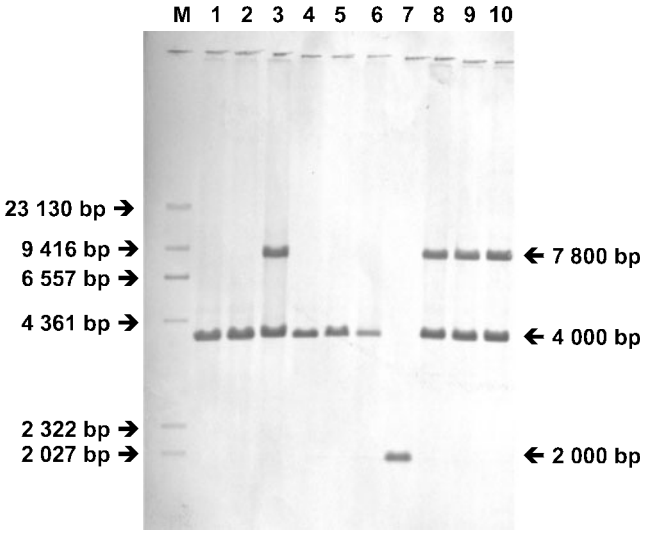


**Fig. 4.** Southern blotting showing the presence of *ptsG* genes in 10 *Mmymy*SC strains from different geographical origins and representing different IS*1296* hybridization patterns determined as described by Cheng *et al.* (1995). The Southern blots were prepared from *Hin*dIII-digested chromosomal DNA, and hybridization was performed with the *ptsG* PCR probe (Table 2). Lanes: 1, strain 6671 isolated in Italy in 1993 (E1*); 2, strain 2091 isolated in France in 1984 (E1*); 3, strain Filfili isolated in Senegal before 1989 (A3*); 4, strain 2022 isolated in France in 1984 (E1*); 5, strain 2117 isolated in France in 1984 (E1*); 6, strain 6364 isolated in Spain in 1991 (E1*); 7, strain 6363 isolated in Spain in 1991 (E1*); 8, strain Afadé isolated in Chad in 1968 (A1*); 9, vaccine strain T1/44/2 isolated in Tanzania in 1952 (A4*); 10, reference strain PG1 (P*) of unknown origin; M, DNA ladder.

622 (from the start codon of MSC_0860; Fig. 5a). No difference was observed in the region between the end of IS*1634*AL and the start codon of MSC_0860, i.e. the putative promoter region of MSC_0860. The nucleotide substitution generates a stop codon (TAA) upstream of the region encoding the 3F3 epitope and a *Tru*9I restriction site that is not present in the 3F3-ON variant. Using the IS*1634*For or PGI1 primers with the PGRev primer, the region encompassing the point mutation was amplified by PCR from DNA of 3F3-ON and 3F3-OFF clonal variants of the Afadé strain. Comparison of the *Tru*9I restriction profiles of the resulting PCR product confirmed the presence of an additional restriction site for 3F3-negative variants (Fig. 5b).

## DISCUSSION

### A new antigenic variation in *Mmymy* SC

A novel manifestation of phase variation occurs in *Mmymy* SC. Surface-exposed proteins which bind *Mmymy* SC-specific mAb 3F3 were proved to undergo low-frequency ON/OFF switching. Variable staining intensity during colony immunostaining was also noted within the cell population of 3F3-ON type variants. Furthermore, colonies showing strong or weak staining coexisted with sectored colonies in which certain sectors were more strongly stained than others (Fig. 1, strain T1/44/2). Sectored colonies are typically encountered with surface antigens which exhibit a high frequency of intraclonal protein variation. The immunostained sectors result from mutations that have induced a modification in the expression or accessibility of the target antigen during colony growth. This suggests that, in addition to ON/OFF switching, variations in size or number may also occur within the PtsG proteins. However, Western blotting analysis of the strongly and weakly stained variants obtained by subcloning within ON-type variants did not confirm this hypothesis, as no significant differences in size were identified between variants (data not shown). Further studies are needed to explain the variation in staining intensity observed during colony immunostaining.

The phage-display library (Persson *et al.*, 2002) and genome database (Westberg *et al.*, 2004) have already proved efficient in identifying a particular protein epitope and its corresponding gene. However, there is an obvious risk of creating false epitopes in fusion proteins (Fehrsen & du Plessis, 1999; Murthy *et al.*, 1999). The specific recognition by mAb 3F3 of a synthetic oligopeptide deduced from a phage-display-selected DNA sequence abolished any remaining doubts about the reliability of the selection during pannings and confirmed identification of the true gene encoding the proteins which bind mAb 3F3. This gene was identified as *ptsG*, which encodes a putative glucose PTS permease. Phase variation in mycoplasma transporters has so far only been documented for one subunit in an ABC transporter of *Mycoplasma fermentans* (Theiss & Wise, 1997). These authors speculated that variation might not only have consequences for immune evasion, but also provide alternative transport capacities through the use of different subsets of the same genes.

### Genetics of glucose PTS

Two *ptsG* genes, MSC_0860 and MSC_0873, potentially code for the proteins recognized by 3F3 mAb and show homology with the glucose PTS permeases. The *ptsG* gene of *Mmymy* SC contained seven alternative initiation codons, and two of the theoretical ORFs were predicted to contain a signal sequence, suggesting the putative expression of at least two surface-exposed proteins of different sizes. Such a coordinate phase variation of two distinct surface antigens encoded by a single gene has also been reported in *Mycoplasma synoviae* (Noormohammadi *et al.*, 1998). These genes are in a duplicated region containing several copies of IS*1634*. Sequence analysis showed that IS*1634*CE, which separates the two chromosomal repeats, is bordered by two different sequences, each corresponding to the direct repeats found for the insertion sequence bordering the chromosomal DNA repeat (Fig. 3). Such chimerical organization leads to the conclusion that

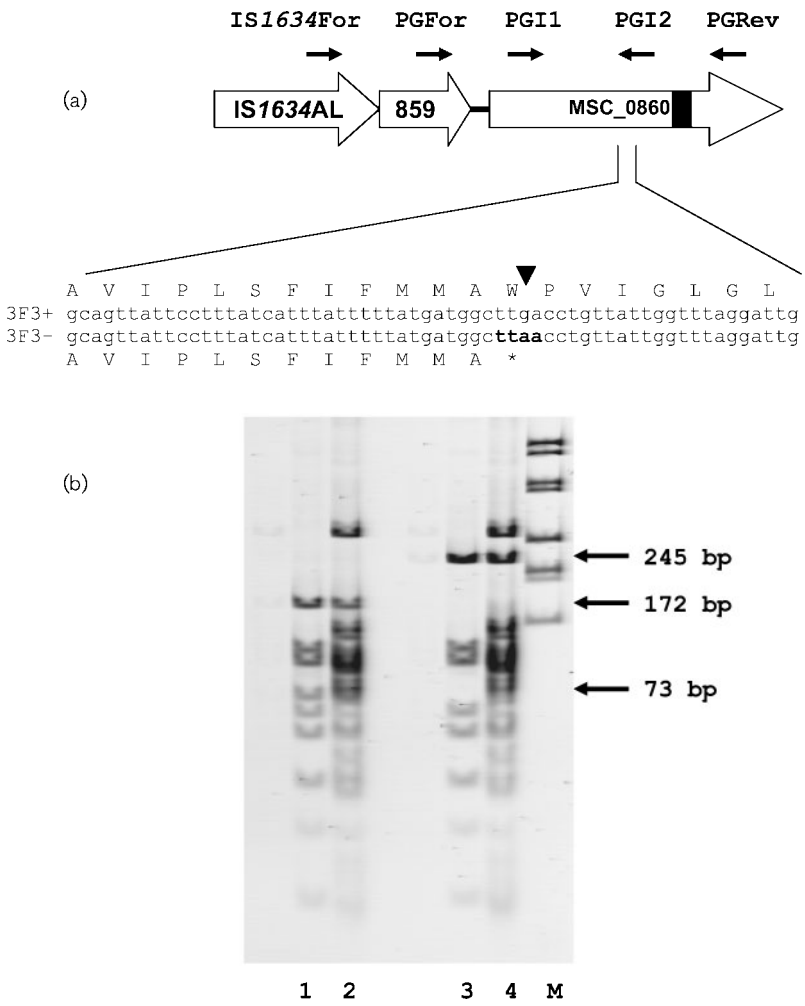


**Fig. 5.** (a) Schematic representation of IS*1634*AL and MSC_0860 gene organization. The black box represents the region encoding the 3F3 epitope. Primers used for amplification and sequencing are shown. The sequence alignment represents the region of the MSC_0860 gene carrying the mutation between the 3F3-ON variant (upper line) and the 3F3-OFF variant (lower line). The arrowhead indicates the nucleotide that differs between the two sequences. Bold type indicates the presence of the *Tru*9I restriction site. (b) *Tru*9I restriction profile of the PCR product obtained with primers PGI1 and PGRev (lanes 1 and 3) and primers IS*1634*For and PGRev (lanes 2 and 4) from genomic DNA extracted from the 3F3-OFF variant (lanes 1 and 2) or the 3F3-ON variant (lanes 3 and 4). M, DNA ladder.

IS*1634*CE was involved in duplication of the *ptsG* region. The insertion of IS*1634*AX and IS*1634*AZ inside the chromosomal repeats occurred after the duplication of the *ptsG* region, as shown by different direct repeats bordering both insertion sequences.

The two regions could be distinguished by Southern blot hybridization. The copy containing MSC_0860 is carried by a 4 kb fragment and the 7·8 kb fragment contains the MSC_0873 gene. We showed that the European strains lack the 7·8 kb fragment, i.e. the MSC_0873 copy. As strains with only the MSC_0860 copy expressed the epitope recognized by 3F3 mAb, we concluded that the expression of MSC_0860 is sufficient to have the protein recognized by 3F3 mAb. The 3F3-OFF type does not express MSC_0860 and MSC_0873. Whether such bacteria are still able to use glucose or not remains unknown due to the presence of another *ptsG* gene, MSC_0054.

The three putative PtsG proteins show a structure atypical for mycoplasmas. Up till now, an IICBA organization has been described for mycoplasmas, but this association of the IIA domain with IICB was not found for *Mmymy* SC. Two genes, MSC_0274 and MSC_0394, encoding hypothetical IIA protein, were found in *Mmymy* SC (Westberg *et al.*, 2004). The protein deduced from MSC_0274 is similar to the IIA of *M. penetrans* (accession no. NP_757834·1) and that deduced from MSC_0394 is similar to IIA protein of *Mycoplasma capricolum* (accession no. P45618) and to IIA of *S. citri* (accession no. AAP55649·1). Interestingly, this similarity pattern is the same as that described for IICB proteins. The organization observed for *Mmymy* SC was also described for a phytopathogenic mollicute, *S. citri* (André *et al.*, 2003). It was suggested that this structure (IIA + IICB) is common to all bacteria that have arthropods as hosts, but *Mmymy* SC has no arthropods as hosts.

Preliminary results indicate that the genetic mechanism involved in ON-to-OFF transition is a base substitution (G to A) leading to the creation of a stop codon uspstream of the sequence encoding the region recognized by mAb 3F3. This substitution directly generates a stop codon, without a frameshift. This is an unusual mechanism of antigenic variation in *Mmymy* SC. However, a similar mechanism was observed for the *gapA* gene of *Mycoplasma gallisepticum* (Winner *et al.*, 2003). Data collected in our study revealed that the 3F3-OFF variant of the Afadé strain spontaneously generates a 3F3-ON variant at high frequency (data not

show), indicating that this mutation is reversible, as was observed for the *gapA* gene of *M. gallisepticum*.

### Distribution of the *ptsG* gene within the *Mmymy* SC biotype

All the molecular analyses clearly distinguish the European strains from those of other geographical origins (Africa, Australia and Asia) (Cheng *et al.*, 1995; Poumarat & Solsona, 1995; Lorenzon *et al.*, 2003). The European strains lack a genomic segment of 8·84 kb that is present in all the other strains (Vilei *et al.*, 2000). As this 8·84 kb deleted segment does not include a copy of the *ptsG* gene, a new genetic specificity has been identified within the European cluster. European strains have a single copy of the *ptsG* gene, whereas all the other *Mmymy* SC strains have two copies. Within the African cluster, a large chromosomal segment including the *ptsG* gene has been duplicated at some stage in evolution. The European PO-67 strain showed a unique pattern intermediate between the two main *Mmymy* SC clusters, i.e. an IS*1296* profile typical of the African cluster, but harbouring only one *ptsG* gene copy like the European cluster. The PO-67 strain is the oldest European strain kept in collection and was isolated from a French outbreak in 1967 (Table 1). Seventeen severe CBPP outbreaks occurred during that year in the eastern part of the French Pyrenees and 500 bovines with clinical symptoms were slaughtered (Anonymous, 1967). The PO-67 strain had never been included in previous studies of molecular epidemiology, except in a recent multilocus sequence analysis study (Lorenzon *et al.*, 2003) that also clearly classified the PO-67 strain in the European group, even though it did not exhibit the 8·84 kb deletion. This observation suggests that the deletion may be a recent feature of *Mmymy* SC evolution in Europe, and that the PO-67 strain may be reminiscent of the highly virulent form of CBPP that affected Europe in the nineteenth century. As the PO-67 strain did not show either the duplication or the deletion, it might be closely related to the unknown ancestor of all *Mmymy* SC strains.

### Relation between PtsG and host

It was previously shown that mAb 3F3 targets a specific *Mmymy* SC epitope that is recognized by sera from CBPP-infected animals (Brocchi *et al.*, 1993). The mAb 117/5 that is used in a diagnostic ELISA (Le Goff & Thiaucourt, 1998) was tested on dot blots of the 3F3 synthetic peptide. Intense positive staining of the dot blots and confirmatory Western blottings with whole-cell lysates of the strain Afadé, in which mAbs 3F3 and 117/5 were compared, showed that these two antibodies target the same epitope (data not shown). Previous studies with mAb 117/5 can, therefore, give us a hint of the expression of PtsG *in vivo*. Amaro *et al.* (2000) performed immunohistochemistry in tissues from CBPP-infected animals at different stages of infection with mAb 117/5 and a polyclonal reference serum raised against *Mmymy* SC. mAb 117/5 and the polyclonal serum targeted *Mmymy* SC cells in all infected tissues, but mAb 117/5 did not react with *Mmymy* SC cells localized in the follicular germinal centre of lymph nodes from animals with chronic lesions, although these were detected by the polyclonal. These results may indicate that this phase variation of PtsG proteins can occur *in vivo*, and might suggest a biological transformation that results in the establishment of a latent maintenance stage that circumvents the killing action of the host defence.

Colony immunostaining was used to compare the relative phenotypic proportions of the ON and OFF types for expanded cultures from i) fourth passage without cloning of the Afadé strain isolated from an acute form of CBPP, ii) a T1/44/2 strain vaccine vial and iii) a KH3J collection vial. The proportion of negative colonies varied greatly: 0·01 % for the Afadé strain, 50 % for the T1/44/2 strain and 99·999 % for the KH3J strain. These results suggest a potential relationship between the level of PtsG protein expression and the respective virulence (high, moderate and non-virulent) of these three strains. However, analysis of the phase variation of the PtsG protein did not reveal any difference between the Afadé and T1/44/2 strains other than a constant extremely low ON/OFF reversion rate in T1/44/2. Surprisingly, the vaccine was shown to consist of an equal mixture of ON-type and OFF-type 3F3 phenotypes. Antigenically distinct subpopulations in vaccine seed strains have already been reported (Rweyemamu *et al.*, 1995). Further investigations would be useful to see if variations in the proportions of 3F3-ON and 3F3-OFF phenotypes in vaccine culture are correlated with virulence and the protective efficacy of the vaccine.

In conclusion, this report provides evidence for variation affecting a putative membrane transport system in mycoplasmas. This variation in a putative glucose-specific PTS of *Mmymy* SC involves a single base substitution leading to the truncation of the protein. Further investigations are required to see if such variation provides alternative transport capacities and also to see if it results in a regulation of substrate import.

## ACKNOWLEDGEMENTS

We express our sincere gratitude to Patrice Cuchet and Evelyne Guérin for skilful technical assistance and to Diana Warwick for reviewing the English. The authors would also like to thank Dr François Thiaucourt from CIRAD-EMVT for providing us with the monoclonal antibody 117/5 and for helpful discussion. This work was funded by grants from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning and by the Agence Française de Sécurité Sanitaire des Aliments (research programme no. PR-2002/9).

## REFERENCES

**Abdo, E. M., Nicolet, J., Miserez, R., Goncalves, R., Regalla, J., Griot, C., Bensaide, A., Krampe, M. & Frey, J. (1998).** Humoral and bronchial immune responses in cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* small colony type. *Vet Microbiol* **59**, 109–122.

**Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997).** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

**Amaro, A., Ferronha, H., Correia, I. & Thiaucourt, F. (2000).** Localization of *Mycoplasma mycoides* subsp. *mycoides* SC surface antigen in lungs lesions and lymph nodes of animals with CBPP. In *COST 826, Mycoplasmas of Ruminants: Pathogenicity, Diagnostics, Epidemiology and Molecular Genetics*, pp. 159–162. Edited by D. Bergonier, X. Berthelot & J. Frey. Luxemburg: European Community.

**André, A., Maccheroni, W., Doignon, F., Garnier, M. & Renaudin, J. (2003).** Glucose and trehalose PTS permeases of *Spiroplasma citri* probably share a single IIA domain, enabling the spiroplasma to adapt quickly to carbohydrate changes in its environment. *Microbiology* **149**, 2687–2696.

**Anonymous (1967).** Evolution de la péripneumonie contagieuse des bovidés dans les différents pays et moyens mis en oeuvre pour sa prophylaxie. *Rev Sci Tech* **67**, 258–259.

**Belli, P., Poumarat, F., Perrin, M., Longchambon, D. & Martel, J. L. (1989).** Reproduction expérimentale et évolution de la péripneumonie contagieuse bovine dans un groupe de bovins et de caprins; aspects anatomo-cliniques. *Rev Elev Med Vet Pays Trop* **42**, 349–356.

**Bhugra, B., Voelker, L. L., Zou, N., Yu, H. & Dybvig, K. (1995).** Mechanism of antigenic variation in *Mycoplasma pulmonis*: interwoven, site-specific DNA inversions. *Mol Microbiol* **18**, 703–714.

**Boesen, T., Emmersen, J., Jensen, L. T., Ladefoged, S. A., Thorsen, P., Birkelund, S. & Christiansen, G. (1998).** The *Mycoplasma hominis vaa* gene displays a mosaic gene structure. *Mol Microbiol* **29**, 97–110.

**Boguslavsky, S., Menaker, D., Lysnyansky, I., Liu, T., Levisohn, S., Rosengarten, R., Garcia, M. & Yogev, D. (2000).** Molecular characterization of the *Mycoplasma gallisepticum pvpA* gene which encodes a putative variable cytadhesin protein. *Infect Immun* **68**, 3956–3964.

**Brocchi, E., Gamba, D., Poumarat, F., Martel, J.-L. & De Simone, F. (1993).** Improvements in the diagnosis of contagious pleuropneumonia through the use of monoclonal antibodies. *Rev Sci Tech* **12**, 559–570.

**Cheng, X., Nicolet, J., Poumarat, F., Regalla, J., Thiaucourt, F. & Frey, J. (1995).** Insertion element IS*1296* in *Mycoplasma mycoides* subsp. *mycoides* small colony identifies a European clonal line distinct from African and Australian strains. *Microbiology* **141**, 3221–3228.

**Citti, C. & Rosengarten, R. (1997).** Mycoplasma genetic variation and its implication for pathogenesis. *Wien Klin Wochenschr* **109**, 562–568.

**Citti, C., Kim, M. F. & Wise, K. S. (1997).** Elongated versions of Vlp surface lipoproteins protect *Mycoplasma hyorhinis* escape variants from growth-inhibiting host antibodies. *Infect Immun* **65**, 1773–1785.

**Citti, C., Watson-McKown, R., Droesse, M. & Wise, K. S. (2000).** Gene families encoding phase- and size-variable surface lipoproteins of *Mycoplasma hyorhinis*. *J Bacteriol* **182**, 1356–1363.

**Claros, M. G. & von Heijne, G. (1994).** TopPred II: an improved software for membrane protein structure predictions. *Comput Appl Biosci* **10**, 685–686.

**Corpet, F. (1988).** Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**, 10881–10890.

**Corpet, F., Gouzy, J. & Kahn, D. (1999).** Recent improvements of the ProDom database of protein domain families. *Nucleic Acids Res* **27**, 263–267.

**Costas, M., Leach, R. H. & Mitchelmore, D. L. (1987).** Numerical analysis of PAGE protein patterns and the taxonomic relationships within the "*Mycoplasma mycoides* cluster". *J Gen Microbiol* **133**, 3319–3329.

**Fehrsen, J. & du Plessis, D. H. (1999).** Cross-reactive epitope mimics in fragmented-genome phage display library derived from the rickettsia, *Cowdria ruminantium*. *Immunotechnology* **4**, 175–184.

**FlitmanTene, R., Levisohn, S., Lysnyansky, I., Rapoport, E. & Yogev, D. (2000).** A chromosomal region of *Mycoplasma agalactiae* containing *vsp*-related genes undergoes *in vivo* rearrangement in naturally infected animals. *FEMS Microbiol Lett* **191**, 205–212.

**Frey, J., Cheng, X., Kuhnert, P. & Nicolet, J. (1995).** Identification and characterization of IS*1296* in *Mycoplasma mycoides* subsp. *mycoides* SC and presence in related mycoplasmas. *Gene* **160**, 95–100.

**Glew, M. D., Baseggio, N., Markham, P. F., Browning, G. F. & Walker, I. D. (1998).** Expression of the *pMGA* genes of *Mycoplasma gallisepticum* is controlled by variation in the GAA trinucleotide repeat lengths within the 5′ noncoding regions. *Infect Immun* **66**, 5833–5841.

**Glew, M. D., Papazisi, L., Poumarat, F., Bergonier, D., Rosengarten, R. & Citti, C. (2000).** Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. *Infect Immun* **68**, 4539–4548.

**Glew, M. D., Marenda, M., Rosengarten, R. & Citti, C. (2002).** Surface diversity in *Mycoplasma agalactiae* is driven by site-specific DNA inversions within the *vpma* multigene locus. *J Bacteriol* **184**, 5987–5998.

**Horino, A., Sasaki, Y., Sasaki, T. & Kenri, T. (2003).** Multiple promoter inversions generate surface antigenic variation in *Mycoplasma penetrans*. *J Bacteriol* **185**, 231–242.

**Jacobsson, K. & Frykberg, L. (1998).** Gene VIII-based, phage-display vectors for selection against complex mixtures of ligands. *BioTechniques* **24**, 294–301.

**Le Goff, C. & Thiaucourt, F. (1998).** A competitive ELISA for the specific diagnosis of contagious bovine pleuropneumonia (CBPP). *Vet Microbiol* **60**, 179–191.

**Le Grand, D., Solsona, M., Rosengarten, R. & Poumarat, F. (1996).** Adaptive surface antigen variation in *Mycoplasma bovis* to the host immune response. *FEMS Microbiol Lett* **144**, 267–275.

**Lorenzon, S., Arzul, I., Peyraud, A., Hendrikx, P. & Thiaucourt, F. (2003).** Molecular epidemiology of contagious bovine pleuropneumonia by multilocus sequence analysis of *Mycoplasma mycoides* subspecies *mycoides* biotype SC strains. *Vet Microbiol* **93**, 319–333.

**Lysnyansky, I., Sachse, K., Rosenbusch, R., Levisohn, S. & Yogev, D. (1999).** The *vsp* locus of *Mycoplasma bovis*: gene organization and structural features. *J Bacteriol* **181**, 5734–5741.

**Lysnyansky, I., Ron, Y., Sachse, K. & Yogev, D. (2001a).** Intrachromosomal recombination within the *vsp* locus of *Mycoplasma bovis* generates a chimeric variable surface lipoprotein antigen. *Infect Immun* **69**, 3703–3712.

**Lysnyansky, I., Ron, Y. & Yogev, D. (2001b).** Juxtaposition of an active promoter to *vsp* genes via site-specific DNA inversions generates antigenic variation in *Mycoplasma bovis*. *J Bacteriol* **183**, 5698–5708.

**Markham, P. F., Glew, M. D., Whithear, K. G. & Walker, I. D. (1993).** Molecular cloning of a member of the gene family that encodes pMGA, a hemagglutinin of *Mycoplasma gallisepticum*. *Infect Immun* **61**, 903–909.

**Markham, P. F., Glew, M. D., Sykes, J. E., Bowden, T. R., Pollocks, T. D., Browning, G. F., Whithear, K. G. & Walker, I. D. (1994).** The organisation of the multigene family which encodes the major cell surface protein, pMGA, of *Mycoplasma gallisepticum*. *FEBS Lett* **352**, 347–352.

**Markham, P. F., Duffy, M. F., Glew, M. D. & Browning, G. F. (1999).** A gene family in *Mycoplasma imitans* closely related to the *pMGA* family of *Mycoplasma gallisepticum*. *Microbiology* **145**, 2095–2103.

**Muhlradt, P. F., Kiess, M., Meyer, H., Sussmuth, R. & Jung, G. (1998).** Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. *Infect Immun* **66**, 4804–4810.

**Murthy, K. K., Ekiel, I., Shen, S. H. & Banville, D. (1999).** Fusion proteins could generate false positives in peptide phage display. *BioTechniques* **26**, 142–149.

**Neyrolles, O., Chambaud, I., Ferris, S., Prevost, M. C., Sasaki, T., Montagnier, L. & Blanchard, A. (1999).** Phase variations of the *Mycoplasma penetrans* main surface lipoprotein increase antigenic diversity. *Infect Immun* **67**, 1569–1578.

**Nicholas, R., Bashiruddin, J., Ayling, R. & Miles, R. (2000).** Contagious bovine pleuropneumonia: a review of recent developments. *Vet Bull* **70**, 827–838.

**Nielsen, H., Engelbecht, J., Brunak, S. & von Heijne, G. (1997).** A neural network method for identification of prokaryotes and eucaryotes signal peptides and prediction of their cleavage sites. *Int J Neural Syst* **8**, 581–599.

**Noormohammadi, A. H., Markham, P. F., Whithear, K. G., Walker, I. D., Gurevich, V. A., Ley, D. H. & Browning, G. F. (1997).** *Mycoplasma synoviae* has two distinct phase-variable major membrane antigens one of which is a putative hemagglutinin. *Infect Immun* **65**, 2542–2547.

**Noormohammadi, A. H., Markham, P. F., Duffy, M. F., Whithear, K. G. & Browning, G. F. (1998).** Multigene families encoding the major hemagglutinins in phylogenetically distinct mycoplasmas. *Infect Immun* **66**, 3470–3475.

**Noormohammadi, A. H., Markham, P. F., Kanci, A., Whithear, K. G. & Browning, G. F. (2000).** A novel mechanism for control of antigenic variation in the hemagglutinin gene family of *Mycoplasma synoviae*. *Mol Microbiol* **35**, 911–923.

**Persson, A., Jacobsson, K., Frykberg, L., Johansson, K. E. & Poumarat, F. (2002).** Variable surface protein Vmm of *Mycoplasma mycoides* subsp *mycoides* small colony type. *J Bacteriol* **184**, 3712–3722.

**Poumarat, F. & Solsona, M. (1995).** Molecular epidemiology of *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony, the agent of contagious bovine pleuropneumonia. *Vet Microbiol* **47**, 305–315.

**Poumarat, F., Perrin, B. & Longchambon, D. (1991).** Identification of ruminant mycoplasma by dot-immunobinding on membrane filtration (MF dot). *Vet Microbiol* **29**, 329–338.

**Poumarat, F., Le Grand, D., Solsona, M., Rosengarten, R. & Citti, C. (1999).** Vsp antigens and *vsp*-related DNA sequences in field isolates of *Mycoplasma bovis*. *FEMS Microbiol Lett* **173**, 103–110.

**Reizer, J., Michotey, V., Reizer, A. & Saier, M. H., Jr (1994).** Novel phosphotransferase system genes revealed by bacterial genome analysis: unique, putative fructose- and glucoside-specific systems. *Protein Sci* **3**, 440–450.

**Rosengarten, R. & Wise, K. S. (1990).** Phenotypic switching in Mycoplasmas: phase variation of diverse surface lipoproteins. *Science* **24**, 315–318.

**Rosengarten, R. & Yogev, D. (1996).** Variant colony surface antigenic phenotypes within Mycoplasma strain populations: implications for species identification and strain standardization. *J Clin Microbiol* **34**, 149–158.

**Roske, K., Blanchard, A., Chambaud, I., Citti, C., Helbig, J. H., Prevost, M. C., Rosengarten, R. & Jacobs, E. (2001).** Phase variation among major surface antigens of *Mycoplasma penetrans*. *Infect Immun* **69**, 7642–7651.

**Rweyemamu, M. M., Litamoi, J. & Sylla, D. (1995).** Contagious bovine pleuropneumonia vaccines: the need for improvements. *Rev Sci Tech Off Int Epizoot* **14**, 593–601.

**Rydén, S. M. & Isaksson, L. A. (1984).** A temperature-sensitive mutant of *Escherichia coli* that shows enhanced misreading of UAG/A and increased efficiency for some tRNA nonsense suppressors. *Mol Gen Genet* **193**, 38–45.

**Sachse, K., Helbig, J. L., Lysnyansky, I., Grajetzki, C., Muller, W., Jacobs, E. & Yogev, D. (2000).** Epitope mapping of immunogenic and adhesive structures in repetitive domains of *Mycoplasma bovis* variable surface lipoproteins. *Infect Immun* **68**, 680–687.

**Shen, X. J., Gumulak, J., Yu, H. L., French, C. T., Zou, N. X. & Dybvig, K. (2000).** Gene rearrangements in the *vsa* locus of *Mycoplasma pulmonis*. *J Bacteriol* **182**, 2900–2908.

**Theiss, P. & Wise, K. S. (1997).** Localized frameshift mutation generates selective high-frequency phase variation of a surface lipoprotein encoded by a mycoplasma ABC transporter operon. *J Bacteriol* **179**, 4013–4022.

**Titgemeyer, F., Reizer, J., Reizer, A. & Saier, M. H., Jr (1994).** Evolutionary relationships between sugar kinases and transcriptional repressors in bacteria. *Microbiology* **140**, 2349–2354.

**Tulasne, J. J., Litamoi, J. K., Morein, B., Dedieu, L., Palya, V. J., Yami, M., Abusugra, I., Sylla, D. & Bensaid, A. (1996).** Contagious bovine pleuropneumonia vaccines: the current situation and the need for improvement. *Rev Sci Tech* **15**, 1373–1396.

**Vilei, E. M., Nicolet, J. & Frey, J. (1999).** IS*1634*, a novel insertion element creating long, variable-length direct repeats which is specific for *Mycoplasma mycoides* subsp. *mycoides* small-colony type. *J Bacteriol* **181**, 1319–1323.

**Vilei, E. M., Abdo, E. M., Nicolet, J., Botelho, A., Goncalves, R. & Frey, J. (2000).** Genomic and antigenic differences between the European and African/Australian clusters of *Mycoplasma mycoides* subsp *mycoides* SC. *Microbiology* **146**, 477–486.

**Washburn, L. R., Hirsch, S. & Voelker, L. L. (1993).** Mechanisms of attachment of *Mycoplasma arthritidis* to host cells in vitro. *Infect Immun* **61**, 2670–2680.

**Washburn, L. R., Weaver, K. E., Weaver, E. J., Donelan, W. & Alsheboul, S. (1998).** Molecular characterization of *Mycoplasma* arthritidis variable surface protein MAA2. *Infect Immun* **66**, 2576–2586.

**Westberg, J., Persson, A., Holmberg, A., Goesmann, A., Lundeberg, J., Johansson, K. E., Pettersson, B. & Uhlén, M. (2004).** The genome sequence of *Mycoplasma mycoides* subsp. *mycoides* type strain PG1$^T$, the causative agent of contagious bovine pleuropneumonia (CBPP). *Genome Res* **14**, 221–227.

**Winner, F., Markova, I., Much, P., Lugmair, A., SiebertGulle, K., Vogl, G., Rosengarten, R. & Citti, C. (2003).** Phenotypic switching in *Mycoplasma gallisepticum* hemadsorption is governed by a high-frequency, reversible point mutation. *Infect Immun* **71**, 1265–1273.

**Yogev, D., Rosengarten, R., Watson-McKown, R. & Wise, K. (1991).** Molecular basis of *Mycoplasma* surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5′ regulatory sequences. *EMBO J* **10**, 4069–4079.

**Yogev, D., Watson-McKown, R., Rosengarten, R., Im, J. & Wise, K. S. (1995).** Increased structural and combinatorial diversity in an extended family of genes encoding Vlp surface proteins of *Mycoplasma hyorhinis*. *J Bacteriol* **177**, 5636–5643.

**Zhang, Q. & Wise, K. (1996).** Molecular basis of size and antigenic variation of a *Mycoplasma hominis* adhesin encoded by divergent *vaa* genes. *Infect Immun* **64**, 2737–2744.

**Zhang, Q. & Wise, K. (1997).** Localized reversible frameshift mutation in an adhesin gene confers a phase-variable adherence phenotype in mycoplasma. *Mol Microbiol* **25**, 859–869.

**Zhang, Q. J. & Wise, K. S. (2001).** Coupled phase-variable expression and epitope masking of selective surface lipoproteins increase surface phenotypic diversity in *Mycoplasma hominis*. *Infect Immun* **69**, 5177–5181.

**Zheng, X., Teng, L.-J., Watson, H. L., Glass, J. I., Blanchard, A. & Cassel, G. H. (1995).** Small repeating units within the *Ureaplasma urealyticum* MB antigen gene encode serovar specificity and are associated with antigen size variation. *Infect Immun* **63**, 891–898.