Genomic and antigenic differences between the European and African/Australian clusters of *Mycoplasma mycoides* subsp. *mycoides* SC

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***Mycoplasma mycoides* subsp. *mycoides* small-colony type (SC), the aetiological agent of contagious bovine pleuropneumonia (CBPP), can be grouped into two major, epidemiologically distinct, clusters. One cluster contains strains isolated from different European countries since 1980 and a second cluster contains African and Australian strains collected over the last 50 years. Genetic analysis of representative strains from the two clusters revealed a genomic segment of 8.84 kb, located close to a copy of IS1296, which is present in all strains of the African cluster but lacking in all strains of the European cluster. This segment contains a copy of IS1634, a gene for a potential lipoprotein, *lppB*, open reading frames encoding a putative surface-located membrane protein and a hypothetical proline-rich membrane protein, and two open reading frames showing similarity to putative ABC transporters. The product of the *lppB* gene, lipoprotein B (LppB), has an apparent molecular mass of 70 kDa and was shown to be surface located. It is detected with monospecific antibodies in all strains of the African cluster tested, but not in European-cluster strains. DNA sequence analysis of the splicing site at which European strains differ from African-cluster strains by the lack of the 8.84 kb segment showed that the European cluster has arisen by deletion from a strain of the African cluster. Hence, *M. mycoides* subsp. *mycoides* SC strains isolated in different European countries from the newly reemerging outbreaks of CBPP, which occurred after the eradication of the epizootic in Europe in the middle of the 20th century, represent a phylogenetically newer cluster that has been derived from a strain of the older cluster of *M. mycoides* subsp. *mycoides* SC which is still endemic on the African continent.**

Keywords: contagious bovine pleuropneumonia, virulence, epidemiology, lipoprotein, genomic deletion

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is caused by *Mycoplasma mycoides* subsp. *mycoides* SC, a bac-

Abbreviations: ABC, ATP-binding cassette; CBPP, contagious bovine pleuropneumonia; DIG, digoxigenin; SC, small-colony type.

The GenBank accession numbers for the nucleotide sequences determined in this work are: AF165134 for the 3.4 kb *Hind*III fragment from *M. mycoides* subsp. *mycoides* SC strain L2; AF165135 for the analogous locus in strain Afadé (containing *lppB* and IS1634); and AF1651136 for the DNA segment containing *lppB*[MmymyLC] and ORF6[MmymyLC] from *M. mycoides* subsp. *mycoides* LC strain Y-goat.

terium belonging to the class *Mollicutes*. It is one of the most serious cattle diseases in Africa, with a mortality rate of 30–80% (Houshaymi *et al.*, 1997). CBPP was endemic in many parts of the world during the 19th century. The disease was never eradicated from Africa and is still endemic there. However, CBPP was eradicated from North America, Australia and Europe in the first half of the 20th century (Egwu *et al.*, 1996). It has reemerged in Southern Europe within the last two decades, thus giving rise to doubts about the efficacy of the control strategies used so far. Epidemiological and clinical observations indicate that the European outbreaks of CBPP are less virulent than the disease

encountered in Africa. Furthermore, CBPP in Europe seems to be far more insidious, as it is usually chronic, and affected cattle show few distinctive clinical signs and rarely die (Nicholas *et al.*, 1996). This might be due to various factors, including climate, nutrition and animal husbandry. However, recent work has indicated that variations in the infectious agent itself might be the origin of the differences observed in virulence of *M. mycoides* subsp. *mycoides* SC in Europe (Houshaymi *et al.*, 1997; Abdo *et al.*, 1998). In controlled experimental infections of cattle with European and African strains of *M. mycoides* subsp. *mycoides* SC, the strain L2, a representative of the recently isolated European strains, was less virulent than the African strain Afadé (Abdo *et al.*, 1998).

In a broad molecular epidemiological study of *M. mycoides* subsp. *mycoides* SC strains from various continents and countries using fingerprinting with the insertion element IS1296, two different clusters of strains were identified: an African cluster, including strains from endemic African countries and historical Australian strains; and a European cluster containing strains isolated since 1980 from outbreaks in four different European countries (Cheng *et al.*, 1995). Characteristically, strains of the European cluster have a *Hind*III fragment of 3.4 kb detected by the IS1296 probe, which is absent in African strains. African-cluster strains, in contrast, had a 4.4 kb *Hind*III fragment which was not found in the European strains (Cheng *et al.*, 1995) (see also Fig. 1). This clustering was confirmed by typing the strains with the most recently discovered insertion element, IS1634 (Vilei *et al.*, 1999), and this also confirmed that the outbreaks in Europe since 1980 are of different origin than those in Africa. Examination of the antigenic profiles of *M. mycoides* subsp. *mycoides* SC strains using immunoblot analysis of total cell proteins also revealed differences between European and African strains (Poumarat & Solsona, 1994). In particular, European strains were found to lack a dominant antigen with an apparent molecular mass of 70–71.5 kDa which was present in all African and Australian strains (Gonçalves *et al.*, 1998).

In order to reveal the genetic basis for these antigenic differences between African and European strains and to obtain further insights into the evolutionary mechanisms that led to the strains causing the newly reemerging outbreaks of CBPP in Europe, we cloned and characterized a genetic locus which contains a significant difference between the two main clusters of *M. mycoides* subsp. *mycoides* SC.

METHODS

Strains, growth conditions and DNA extraction. Strains of *M. mycoides* subsp. *mycoides* SC and of the other *Mycoplasma* species used in this study are listed in Table 1. Mycoplasmas were cultured in a standard mycoplasma medium at 37 °C (Bannerman & Nicolet, 1971) to a density of 10^8 – 10^9 cells ml⁻¹. Crude lysates were prepared by addition of GES buffer (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% N-

lauroylsarcosine) and DNA extractions were performed as previously described (Cheng *et al.*, 1995).

Southern blot analysis. Genomic mycoplasma DNA was digested with *Hind*III, and fragments were separated by electrophoresis through a 0.7% agarose gel, then transferred onto positively charged nylon membrane (Boehringer Mannheim). The membrane was briefly rinsed with 1 × SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.7) and DNA was denatured at 80 °C under vacuum for 30 min (Ausubel *et al.*, 1990). The membrane was incubated in 10 ml hybridization buffer, consisting of 5 × SSC, 0.02% SDS, 0.1% N-lauroylsarcosine and 1% blocking reagent (Boehringer Mannheim), at 68 °C for 2 h and then incubated in 5 ml hybridization buffer containing 3 µl digoxigenin-11-dUTP (DIG)-labelled IS1296 probe, prepared as described previously (Cheng *et al.*, 1995), for 15 h at 68 °C. It was washed twice for 5 min at room temperature with 2 × SSC containing 0.1% SDS, and twice for 15 min at room temperature with 0.2 × SSC containing 0.1% SDS. The hybridized DIG-labelled probe was detected using phosphatase-labelled anti-DIG antibodies (Boehringer Mannheim) as described in the manufacturer's protocol.

Cloning and sequencing strategies. A partial genomic library of *M. mycoides* subsp. *mycoides* SC strain L2 was made by cloning total DNA digested with *Hind*III into the *Hind*III site of vector pBluescriptII SK(–) (Stratagene). Clones were selected for the presence of IS1296 by colony screening with the DIG-labelled IS1296 probe. Plasmid DNA of the positive colonies was isolated using the QIAprep Spin Plasmid kit (Qiagen). Double-stranded nested deletion, using exonuclease III (Pharmacia Biotech), was carried out following the manufacturer's protocol to sequence cloned DNA fragments. Sequencing was performed with a DNA Sequenator AB310 and the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer) using primers complementary to the T3 and T7 promoters of the vector. Comparisons of DNA sequences and their deduced amino acid sequences with the EMBL/GenBank and NBRF databases were performed using the programs BLASTN, BLASTX and BLASTP (Altschul *et al.*, 1990). Analysis of protein sequences for characteristic motifs was done using the programs PROSITE (Bairoch *et al.*, 1995) and SignalIP (Nielsen *et al.*, 1997).

PCR reactions. Oligonucleotide primer sequences used for PCR amplifications and their corresponding annealing temperatures are given in Table 2. The PCR reactions were carried out in a DNA thermal cycler Gene Amp 9600 (Perkin Elmer) in 20 µl reaction mix (50 mM Tris/HCl, pH 9.2, 1.75 mM MgCl₂, 16 mM (NH₄)₂SO₄, 350 µM of each dNTP, and 300 nM forward and reverse primers) using as template 2–10 ng purified mycoplasma DNA. To each reaction, 2.5 units *Taq* DNA polymerase or 1.75 units of a mixture of *Taq* DNA and *Pwo* DNA polymerase (Expand Long Template PCR System kit, Boehringer Mannheim) were added. The latter was used for amplification of long DNA fragments (> 3 kb) or for DNA segments that were used for cloning and for sequencing. The samples were subjected to 30 cycles of amplification consisting of 30 s at 94 °C, 30 s at 48 °C and elongation at 68 °C according to the length of the amplicon desired. The PCR amplification products were analysed by electrophoresis through 0.7% agarose gels and visualized after staining with ethidium bromide on a UV transilluminator (Ausubel *et al.*, 1990).

Expression and purification of His-tailed LppB. Primers ORF4N(KpnI) and ORF4C (Table 2), containing a *Kpn*I and a *Bam*HI restriction site overhang, respectively, and genomic

Table 1. *Mycoplasma* strains used

<i>Mycoplasma</i> species	Strain*	Origin	Year isolated	Host	PCR product (kb)†
<i>M. mycoides</i> subsp. <i>mycoides</i> SC	PG1 ^a		1931	Cattle/type strain	11·3
	2059 ^b	Spain	1984	Cattle/lung	2·5
	B773/125 ^c	Portugal	1991	Cattle/semens	2·5
	C305 ^c	Portugal	1993	Goat/lung	2·5
	O326 ^c	Portugal	1993	Sheep/milk	2·5
	PO 2 ^d	France	1980	Cattle/lung	2·5
	2022 ^b	France	1984	Cattle/lung	2·5
	L2 ^{e,g}	Italy	1993	Cattle/lung	2·5
	402 ^b	Italy	1990	Cattle/lung	2·5
	6479 ^b	Italy	1992	Cattle/lung	2·5
	Afadé ^{d,g,h}	Cameroon	1968	Cattle/lung	11·3
	Fatick ^d	Senegal	1968	Cattle	11·3
	B17 ^d	Chad	1967	Zebu	11·3
	9050-529/1 ^d	Ivory Coast	1990	Cattle	11·3
	91130 ^d	Central African Republic	1991	Cattle	9·0
	94111 ^d	Rwanda	1994	Cattle	11·3
	95014 ^d	Tanzania	1995	Cattle	11·3
	T1/44 ^d	Tanzania	1952	Cattle/vaccine strain	11·3
	T1/Sr50 ^d	Tanzania	1952	Cattle/vaccine strain	11·3
	Gladysdale ^f	Australia		Cattle	11·3
	V5 ^f	Australia	1965	Cattle/vaccine strain	11·3
<i>M. mycoides</i> subsp. <i>mycoides</i> LC	Y-goat ^a	Australia		Goat/type strain	—
<i>M. mycoides</i> subsp. <i>capri</i>	PG3 ^a			Goat/type strain	—
<i>Mycoplasma</i> sp. bovine group 7	PG50 ^a	Australia		Cattle/reference strain	—
<i>M. capricolum</i> subsp. <i>capricolum</i>	California kid ^a	California		Goat/type strain	—
<i>M. capricolum</i> subsp. <i>capripneumoniae</i>	F38 ^a	Kenya		Goat/type strain	—

* Collection: ^aNational Collection of Type Cultures (NCTC), PHLS, London, UK; ^bLaboratoire de Pathologie Bovine, Lyon, France; ^cLaboratório Nacional de Veterinária, Lisbon, Portugal; ^dCIRAD-EMVT, Montpellier, France; ^eInstitute for Veterinary Bacteriology, University of Berne, Berne, Switzerland; ^fAustralian Animal Health Laboratory, Geelong, Victoria, Australia; ^gReference strains representing the European- and African-cluster strains; ^hIsolated at Farcha Laboratory, N'Djaména, Chad, from a bovine from Afadé, Northern Cameroon.

† Size of fragment obtained by PCR using primers 3480bp-L and 3480bp-R. —, No amplification.

DNA of strain Afadé were used to amplify the 5'-terminal part of the *lppB* gene, corresponding to amino acids 43–338 (Fig. 2). This region does not contain UGA_{Trp} codons. Plasmid pJFFLppB-His, encoding the polyhistidine-tailed N-terminal part of LppB, was constructed by ligating the amplicon into the *KpnI*–*Bam*HI restriction sites of the T7-promoter-based expression vector pETHIS-1 (Schaller *et al.*, 1999). The plasmid was purified using the QIAprep Spin Plasmid kit, sequenced using primers complementary to the T7 promoter and to the 3'-terminal region flanking the multi-cloning site of the vector to confirm that it contained the expected insert, and transformed into *Escherichia coli* BL21(DE3) cells (Novagen) for expression. One positive clone was inoculated into 50 ml LB broth containing 50 µg ampicillin ml⁻¹ and incubated at

37 °C to an OD₆₀₀ of 0·45, then expression was induced by addition of 1 mM IPTG and incubation continued for another 3 h. The cells were sedimented by centrifugation at 3000 g for 10 min, resuspended in 5 ml PN buffer (50 mM NaH₂PO₄, pH 8, 300 mM NaCl), sonicated with a microtip for 4 min with the power output control at 7 and a duty cycle of 50% (1 s pulses) in a Branson Sonifier 250 (Branson Ultrasonics), and then centrifuged at 15 000 g for 20 min. The supernatant containing the cytosolic fraction was kept, and the pelleted cell debris was resuspended in 5 ml of PN buffer (insoluble fraction). Analysis of the sonicated fractions on SDS/10% acrylamide gels (Laemmli, 1970) showed that the induced protein was in the pellet. Guanidine hydrochloride was added to a final concentration of 6 M to the insoluble fraction and

Table 2. Oligonucleotide primers

Name	Sequence*	Position†	Annealing temp. (°C)‡
3480bp-L	TCTGATTTAGTTGGATTGAGTTCA	1–24	49·6
3480bp-R	GCACCAATGAAGCTAATAGAACA	11329–11306	50·3
7500bp4L	TGATTGAATTTAGCAGCACC	3265–3284	49·0
7500bp4R	TTACTAAATAAACTACCTTTC	9695–9674	46·0
DIG-4L	TCTTCTTCAATACCACTAAC	8081–8100	45·9
ORF4N (KpnI)	GCggtaccACCTTTATCTTTAGCTTTAAGA	9459–9430	47·2§
ORF4C	TCggtaccTAAGTTTTTGAATTCTTGTTT	8555–8583	46·9§

* Lower-case letters indicate nucleotides added to create restriction enzyme recognition sites for cloning.

† Based on nucleotide sequence AF165135 (11·3 kb PCR product from Afadé).

‡ Obtained with the 'PCR primer annealing temperature calculator' developed by J. Boxall on the web page <http://www.res.bbsrc.ac.uk/biochem/oligos/input.html>, by using the parameters 30% as target GC content and 500 bp as target size.

§ Not considering the nucleotides added to create the restriction enzyme recognition sites.

the mixture was loaded onto a prewashed 2·5 ml bed volume Ni/nitrilotriacetic acid/agarose column (Qiagen) and washed once more with 30 ml PNG buffer (50 mM NaH₂PO₄, pH 8, 300 mM NaCl, 6 M guanidine hydrochloride). Step elution of the protein was performed with 10 ml PNG buffer at each different pH (7·0, 6·0, 5·5, 5·0 and 4·5) and fractions of 1 ml were collected. The fractions were dialysed and analysed on SDS/10% acrylamide gels. The purified fusion protein eluted at pH 4·5 was dialysed overnight against PN buffer.

Production of monospecific mouse and rabbit anti-LppB antibodies and immunoblot analysis. Monospecific, polyclonal antibodies directed against LppB were obtained by immunizing mice subcutaneously with 0·5 µg recombinant polyhistidine-tailed LppB protein in 100 µl PN buffer mixed with 100 µl Freund's complete adjuvant (Difco) followed by a booster immunization with the same amount of protein in Freund's incomplete adjuvant (Difco) 3 weeks later. The animals were bled 10 d after the booster immunization. Antisera were prepared from the blood samples and stored at –20 °C. Polyclonal rabbit antiserum directed against LppB was obtained by the same procedure using 50 µg recombinant polyhistidine-tailed LppB protein for each immunization.

Immunoblot analysis was carried out using standard protocols (Ausubel *et al.*, 1990) and phosphatase-conjugated goat anti-mouse IgG + IgM (H + L) (Kirkegaard & Perry Laboratories) at a dilution of 1:2000, or phosphatase-conjugated goat anti-rabbit IgG (H + L) (Kirkegaard & Perry Laboratories) at a dilution of 1:5000, respectively.

Triton X-114 phase partitioning. *M. mycoides* subsp. *mycoides* SC strain Afadé components were separated into hydrophobic and hydrophilic fractions by the Triton X-114 (Fluka) partitioning method as previously described (Cheng *et al.*, 1996). A 50 ml culture of mycoplasmas was grown to the stationary phase and then harvested by centrifugation. The cells were washed three times in TS buffer (10 mM Tris/HCl, pH 7·5 and 150 mM NaCl) and resuspended in 1 ml TS buffer. Prewashed, condensed Triton X-114 was added to the 1 ml sample to give a final concentration of 1% (w/v) and the mixture was incubated for 30 min at 4 °C with gentle rocking. Insoluble components were then removed by centrifugation at 4 °C for 5 min at 13000 g. The Triton X-114-solubilized

material was incubated for 15 min at 37 °C to allow condensation of the detergent phase, which was then separated by centrifugation at 37 °C for 5 min at 13000 g. The lower, detergent phase was adjusted to its original volume with TS buffer without the addition of Triton X-114. The upper, aqueous phase was transferred to a new tube and chilled to 4 °C, then Triton X-114 was added to a final concentration of 1%. This mixture was rocked at 4 °C for 5 min, incubated for 15 min at 37 °C and then centrifuged at 37 °C for 5 min at 13000 g. This cycle was repeated three times to ensure complete removal of hydrophobic fractions from the aqueous phase. Both phases were finally adjusted to the same volume. Samples from the detergent phase, the aqueous phase and whole mycoplasma cells were mixed with protein sample buffer, run on SDS/10% acrylamide gels and blotted onto nitrocellulose. The filter was subsequently used for immunoblotting with the monospecific, polyclonal antibodies directed against LppB.

RESULTS

Cloning and sequencing of the 3·4 kb *Hind*III fragment specific for European-cluster strains of *M. mycoides* subsp. *mycoides* SC

The 3·4 kb genomic *Hind*III fragment carrying IS1296, which is characteristic for European-cluster strains (Cheng *et al.*, 1995), was selected from a library of plasmid clones containing *Hind*III fragments of *M. mycoides* subsp. *mycoides* SC strain L2 using the IS1296 probe. Plasmid pJFFev3·4-L2, containing IS1296 on a 3·4 kb *Hind*III fragment, was chosen (Fig. 1) and sequenced. The cloned fragment was 3414 bp in size, with an A + T content of 75·7 mol%, which is typical for mycoplasma genomes. The fragment contained a copy of IS1296 between bases 266 and 1751 (Fig. 2), which had a few minor differences compared to the copy of IS1296 previously isolated from another locus (Frey *et al.*, 1995) including five nucleotide substitutions, two nucleotide additions and one nucleotide deletion. Furthermore, the 3·4 kb fragment contained an open read-

Fig. 1. Southern blot hybridization of *Hind*III-digested DNA of strains L2 and Afadé with an IS1296 probe. Samples of 500 ng genomic DNA and 100 ng recombinant plasmid pJFFev3-4-L2 were cut with *Hind*III, separated by gel electrophoresis, transferred onto a positively charged nylon membrane and probed with DIG-labelled IS1296. The black arrowhead indicates the IS1296-carrying 3.4 kb *Hind*III fragment specific for the European strains; the open arrowhead indicates the fragment characteristic of the African strains of *M. mycoides* subsp. *mycoides* SC. The recombinant plasmid pJFFev3-4-L2 contains the 3.4 kb *Hind*III fragment cloned from L2. Abbreviations: Eu, Europe; Af, Africa.

ing frame, ORF5, on the minus strand, representing the 3' end of a truncated gene of an analogue of the proline-rich membrane protein of *Enterococcus faecalis* (EMBL/GenBank accession number X96977) (Fig. 2). ORF5 was preceded by ORF2, representing the 5' end of a truncated gene for a putative ATP-binding cassette (ABC) transporter. Furthermore, ORF2 was preceded by a partially overlapping ORF0 encoding another putative ABC transporter (Fig. 2).

Analysis of the analogous genomic locus in the African *M. mycoides* subsp. *mycoides* SC strain Afadé

In order to clone and subsequently analyse the locus analogous to the above-characterized 3.4 kb *Hind*III fragment from strains of the African cluster, PCR amplification using genomic DNA of strain Afadé as template and oligonucleotide primers 3480bp-L and 3480bp-R, complementary to sequences in the 3.4 kb *Hind*III fragment of strain L2, was performed. This PCR amplification resulted in a fragment of about 11.3 kb from strain Afadé and from all other strains of the

African cluster, except strain 91130, which gave a 9.0 kb fragment (Table 1). PCR with these primers using DNA from European strains as template resulted in a 2.5 kb fragment, indicating that European strains were lacking approximately 8.8 kb at this locus. The DNA fragment of 11.3 kb obtained by PCR amplification from strain Afadé was sequenced directly using oligonucleotide primers 3480bp-L and 3480bp-R, and further primers which were subsequently derived from sequence data. Sequence analysis revealed the presence of an 8841 bp DNA fragment in the genome of Afadé which was not present in the clone from the L2 genomic library, while the remaining 2.5 kb of the sequence was the same for both strains. This explained the difference in the above-described PCR reactions, which amplified an 11.3 kb fragment from African-cluster strains and a 2.5 kb fragment from European strains. During sequence analysis we observed that certain sequencing primers gave two superimposed sequences. This was due to the presence of two directly repeated segments of 478 bp flanking a copy of IS1634 (Fig. 2). These repeats are the longest direct repeats created by IS1634 found thus far in *M. mycoides* subsp. *mycoides* SC (Vilei *et al.*, 1999).

The 11.3 kb fragment of strain Afadé contained, in addition to a full copy of IS1634, five different open reading frames. One of them had the structure of a gene encoding a peptide with a typical signal sequence for lipoproteins, but with no homology to any other product deposited in the EMBL/GenBank database. It was designated *lppB* (Fig. 2), following the proposal for nomenclature of lipoproteins in *M. mycoides* (Monnerat *et al.*, 1999). The *lppB* gene encoded a potential lipoprotein precursor of 622 aa. The N-terminal signal sequence had a signal peptidase II cleavage site after amino acid 23. Hence the mature LppB was predicted to contain 599 aa and to have a molecular mass of 67.4 kDa. There were 6 UGA_{Trp} codons in the *lppB* gene. A further open reading frame, ORF6, was found to encode a putative membrane protein of 516 aa with 23% identity in a 320 aa portion to the surface-located membrane protein Lmp3 of *Mycoplasma hominis* (accession number JC6009). Neither *lppB* nor ORF6 was present in European-cluster strains of *M. mycoides* subsp. *mycoides* SC. In addition, the 11.3 kb amplicon from strain Afadé contained a complete ORF5 encoding the analogue of the proline-rich membrane protein of *E. faecalis* on one side of the splice site corresponding to the 8.84 kb deletion found in the European strains, and a complete ORF2 encoding the previously mentioned putative ABC transporter protein on the other side of the splice site (Fig. 2). Moreover, ORF2 was followed by a partially overlapping ORF3 encoding another putative ABC transporter (Fig. 2).

Presence of the *lppB* gene in *M. mycoides* subsp. *mycoides* SC strains

A DIG-labelled probe prepared by PCR with primers DIG-4L and 7500bp4R (Table 2) was used in Southern blot analysis to detect the presence of *lppB* in the

Fig. 2. Genetic map of the locus containing *lppB* in *M. mycoides* subsp. *mycoides* SC. This locus differentiates European strains (upper part; shown for strain L2) from African-cluster strains (middle part; shown for strain Afadé) and the analogous locus in *M. mycoides* subsp. *mycoides* LC strain Y-goat (lower part). Open boxes indicate the copies of the two IS elements IS1296 and IS1634 found in the clones from strains L2 and Afadé. Dotted boxes represent the 478 bp direct repeats of IS1634 in the 8.84 kb segment specific for Afadé. The large arrows indicate open reading frames found in the three DNA sequences. The positions of the oligonucleotide primers used in this work (see Table 1) are depicted as small arrowheads. *Hind*III sites are represented by vertical bars marked H. The segment containing *lppB* and ORF6 analogues in *M. mycoides* subsp. *mycoides* LC strain Y-goat is shown at the bottom. Thin dotted lines align analogous genomic sections between the different strains.

genomic DNA of a selection of strains of *M. mycoides* subsp. *mycoides* SC digested with *Hind*III. None of the European strains reacted with the probe, while the strains belonging to the African cluster showed three predicted DNA fragments (Fig. 3). This result suggested that there are no additional allelic, silent copies of *lppB* present other than the one identified in the African strains. The presence of the three reacting bands of 3.9, 1.0 and 0.4 kb on the Southern blot was explained by the fact that the *lppB* gene has two *Hind*III restriction sites, as shown in Fig. 2. Hence, *lppB* occurs as a single copy in the African strains.

Presence of LppB in *M. mycoides* subsp. *mycoides* SC strains

Triton X-114 phase partitioning was used as a standard method to perform a general hydrophobicity analysis in order to detect lipoproteins (Cheng *et al.*, 1996). In this method, integral hydrophobic membrane proteins are incorporated into the Triton X-114 micelles, while hydrophilic proteins are sequestered in the aqueous phase. LppB from strain Afadé was identified among the proteins of the Triton X-114 phase on immunoblot strips that were incubated with polyclonal, monospecific anti-recombinant LppB-His antibodies (Fig. 4), indi-

cating that LppB is probably membrane-located. This analysis revealed that the monospecific anti-LppB serum reacted with a protein band at approximately 70 kDa, which corresponded to one of the major antigens detected with serum from a cow that was experimentally infected with *M. mycoides* subsp. *mycoides* SC strain Afadé (Abdo *et al.*, 1998). The difference between the calculated molecular mass of 67.4 kDa for LppB and the apparent molecular mass of 70 kDa as seen on SDS-PAGE reflects similar observations made for other mycoplasmal lipoproteins (Frey *et al.*, 1998; Monnerat *et al.*, 1999; Zheng *et al.*, 1995; Proft *et al.*, 1995).

In order to study the expression of the *lppB* gene in *M. mycoides* subsp. *mycoides* SC and to correlate it with the 70–71.5 kDa antigen that was found specifically in African-cluster strains (Gonçalves *et al.*, 1998), we analysed whole-cell antigens of different European and African strains on immunoblots using the monospecific polyclonal anti-LppB antibodies. Immunoblots revealed a strong reaction with an antigen of about 70 kDa only in strains of the African cluster, including vaccine strains and strains from Australia; no reaction was observed with European strains (Fig. 5a). These results revealed that LppB was only expressed by strains of the African cluster and not by European strains, thus supporting the

Fig. 3. Detection of *lppB* in strains of *M. mycoides* subsp. *mycoides* SC. Southern blots of genomic mycoplasma DNA cut with *Hind*III were probed with a DIG-labelled *lppB* probe. The strains are described in Table 1. Abbreviations: T, type strain; Eu, Europe; Af, Africa; Au, Australia; superscript c, caprine strain; superscript o, ovine strain; superscript v, vaccine strain; Std, molecular mass standards (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb). Arrows indicate the three reacting *Hind*III fragments from the African-cluster strains.

suggestion that LppB corresponds to the 70–71.5 kDa antigen of African-cluster strains as described previously (Gonçalves *et al.*, 1998). When the recombinant peptide LppB-His, containing amino acids 43–338 of LppB (calculated molecular mass 38 kDa), was probed with two sera from a comparative experimental infection (Abdo *et al.*, 1998), serum from cow no. 512, which was infected with strain Afadé, was found to contain antibodies which bound LppB-His. The serum from cow no. 502, which was infected with the European strain L2, had no anti-LppB antibodies (Fig. 5b), further confirming the specificity of LppB for strains of the African cluster.

To determine if LppB or antigenically similar proteins were present in the other mycoplasmas of the *M. mycoides* cluster, whole-cell lysates of the type and reference strains were analysed on an immunoblot together with whole-cell lysates of strains PG1, L2 and Afadé of *M. mycoides* subsp. *mycoides* SC. The immunoblot assay revealed strong reactions with 70 kDa proteins in *M. mycoides* subsp. *mycoides* LC (strain Y-goat) and *Mycoplasma* sp. bovine group 7 (PG50), as well as some very weak reactions with the other species (Fig. 6). By analogy with the previously analysed lipoproteins (Monnerat *et al.*, 1999), we propose the designations LppB[MmymyLC] for the lipoprotein B of *M. mycoides* subsp. *mycoides* LC, LppB[Mbgr7] for the lipoprotein B of *Mycoplasma* sp. bovine group 7, and LppB[MmymySC] for the lipoprotein B of *M. mycoides* subsp. *mycoides* SC.

Analysis of *lppB*[MmymyLC] in *M. mycoides* subsp. *mycoides* LC

Analysis of the *lppB* gene of *M. mycoides* subsp. *mycoides* LC was performed by PCR amplification with the oligonucleotide primers ORF4N(KpnI) and 7500bp4L (Table 2), matching the 5'-terminal sequence of *lppB*[MmymySC] and the 3'-terminal sequence of ORF6, respectively (Fig. 2), and using genomic DNA of strain Y-goat as template. This PCR resulted in a 3.9 kb fragment which was subsequently sequenced. Sequence analysis confirmed the presence of the *lppB*[MmymyLC] gene and of an analogue to ORF6 in *M. mycoides* subsp. *mycoides* LC (Fig. 2). However, these two genes were not separated by an insertion element as was the case in strain Afadé of *M. mycoides* subsp. *mycoides* SC (Fig. 2). This illustrates the previous finding that IS1634 is specific for *M. mycoides* subsp. *mycoides* SC (Vilei *et al.*, 1999). Consequently, there was also no direct repeat of 478 bp of the 3' end of the *lppB*[MmymyLC] gene. This 3.9 kb DNA region and the corresponding region of the genome of strain Afadé had 91% identical nucleotides. The LppB[MmymyLC] protein had 90.1% amino acids identical and 92.3% amino acids similar to LppB[MmymySC], explaining the serological cross-reactions shown in Fig. 6. Interestingly, an additional segment of 12 bp, encoding 4 aa, was found in *lppB*[MmymyLC]. Similar additional codons were also found in ORF6 of *M. mycoides* subsp. *mycoides* LC strain Y-goat when compared to ORF6 of *M. mycoides* subsp. *mycoides* SC strain Afadé.

Fig. 4. Triton X-114 phase partitioning of *M. mycoides* subsp. *mycoides* SC strain Afadé. Mycoplasmas in the stationary growth phase were submitted to Triton X-114 fractionation; 10 µl of fractions and total cell proteins were separated by 10% SDS-PAGE, transferred onto a nitrocellulose filter and probed with monospecific LppB antiserum. Lanes: 1, molecular mass standards (New England Biolabs, broad-range marker 77085) 175, 83, 62, 47.5, 32.5 and 25 kDa; 2, total cell proteins; 3, detergent phase; 4, aqueous phase.

DISCUSSION

Previous molecular epidemiological investigations subtyping *M. mycoides* subsp. *mycoides* SC strains using insertion element IS1296 fingerprinting revealed a clear clustering of the strains isolated from the reemerging

outbreaks of CBPP in different European countries, and separated them from strains isolated in Africa and Australia over the last 50 years (Cheng *et al.*, 1995). This difference gained further importance with recent studies indicating that strains belonging to the African cluster are potentially more virulent than the current European strains (Abdo *et al.*, 1998; Houshaymi *et al.*, 1997).

The aim of the present study was to identify major genetic differences between strains of the African and European clusters and to find a correlation with the characteristic antigenic difference between the two clusters, represented by the lack of an antigen at 70–71.5 kDa in European strains, as observed previously (Gonçalves *et al.*, 1998). Sequence comparison of an analogous DNA locus in two representative strains of both clusters showed that the genome of the European-cluster strains lacked a segment of 8.84 kb, compared to strains from the African cluster. A copy of IS1296 was found approximately 600 bp from the locus involving this deletion event. This single difference resulted in two distinct changes in the IS1296-profile between African- and European-cluster strains (a *Hind*III band of 3.4 kb for European strains and a *Hind*III band of 4.4 kb for African strains, as shown in Fig. 1). Characterization of both ends of the deletion site in the African-cluster strains revealed the presence of open reading frames at each side. Both encoded full-length genes, one a putative proline-rich membrane protein and the other a putative ABC transporter protein, the latter belonging to a class of transporters which is abundant in mycoplasmas (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). In contrast, in strains of the European cluster only fragments of each of these open reading frames were found (Fig. 2), indicating that the observed genetic difference

Fig. 5. Distribution of LppB expression in strains of *M. mycoides* subsp. *mycoides* SC. (a) Immunoblot analysis was carried out with 10 µg total antigen per lane. Total antigen of a selection of strains from *M. mycoides* subsp. *mycoides* SC was separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane and probed with the antiserum against LppB. Std, molecular mass standards (New England Biolabs, broad-range marker 77085) 175, 83, 62, 47.5, 32.5 and 25 kDa. Other abbreviations as in Fig. 3. The arrowhead indicates the position of LppB on the immunoblot. (b) One-microgram samples of purified recombinant LppB-His (indicated with an arrowhead) were run on 15% SDS-PAGE and immunoblotted using sera obtained from cows experimentally infected with African strain Afadé (lane A) or European strain L2 (lane B).

Fig. 6. Detection of LppB in type and reference strains of the *M. mycoides* cluster by immunoblotting. Approximately 10 µg total antigen of the type and reference strains of different mycoplasmas of the *M. mycoides* cluster, and of the reference strains of the European cluster (L2) and the African cluster (Afadé) of *M. mycoides* subsp. *mycoides* SC, was reacted with monospecific LppB antiserum.

was caused by a deletion event which gave rise to the cluster of European strains, rather than an insertion event in the African-cluster strains. Hence, our genetic data suggest that the European strains descended from an ancestral strain belonging to the African cluster of *M. mycoides* subsp. *mycoides* SC.

While all European strains analysed by PCR were similar in the vicinity of the deletion, one strain of the African cluster, strain 91130, had a 2·3 kb smaller fragment between the splicing sites compared to the other African strains. This difference corresponds to the absence in strain 91130 of one copy of IS1634 plus the corresponding 478 bp direct repeat, found in all other African strains studied. This reflects previous findings that showed that IS1634 patterns among African field strains varied and suggested that there might be a higher frequency of transposition of IS1634 compared to IS1296.

Among the different open reading frames found on the DNA segment that is specific for African-cluster strains, we identified the gene *lppB*, encoding a potential membrane-associated lipoprotein, as deduced from sequence comparisons and analysis. The *lppB* gene was found as a single copy in the African cluster, while European strains were devoid of this gene. This further supports the hypothesis that the European cluster was derived from the African cluster by genomic deletion. Thus, expression of LppB seems to be a significant phenotypic difference distinguishing the African-cluster strains from the European strains and explains the previously observed difference in the antigen profile at 70–71·5 kDa between different strains of *M. mycoides* subsp. *mycoides* SC (Poumarat & Solsona, 1994; Gonçalves *et al.*, 1998). LppB seems to have homologues in *M. mycoides* subsp. *mycoides* LC and *Mycoplasma*

sp. bovine group 7, as revealed by immunoblot analysis using monospecific antisera directed against recombinant LppB.

It has to be noted that beside African field strains, also strains from Australia, and the African and Australian vaccine strains of *M. mycoides* subsp. *mycoides* SC, possess the *lppB* gene. Historical isolates from Australia were previously shown by IS1296 typing to belong to the same cluster as strains isolated from Africa (Cheng *et al.*, 1995). The presence of the *lppB* gene in vaccine strains and the fact that the LppB protein is expressed in these strains indicates that their virulence is attenuated at another genetic locus.

Membrane lipoproteins of several pathogenic mycoplasmas have been shown to induce blastogenesis and secretion of proinflammatory cytokines by a mechanism distinct from that of lipopolysaccharides, and hence may be important mycoplasma virulence factors (Brenner *et al.*, 1997; Herbelin *et al.*, 1994; Mühlradt & Frisch, 1994; Rawadi & Roman-Roman, 1996). In addition they play an important role in the stability and integrity of the fragile mycoplasma membrane (Razin *et al.*, 1998). LppB therefore might contribute indirectly to the virulence of *M. mycoides* subsp. *mycoides* SC. However, it remains to be determined exactly how the lack of LppB might be responsible for the lower virulence and, in particular, the lower infectivity of European strains, compared to African cluster strains, as observed in comparative experimental infections (Abdo *et al.*, 1998).

In addition to the *lppB* gene, the strains of the European cluster lack several other genes, including putative ABC transporter genes, which might also be involved in the reduced pathogenicity of these strains. Such an effect of putative ABC transporters on virulence was postulated for *M. hyopneumoniae* (Blanchard *et al.*, 1996).

In summary, our results show that the current European strains of *M. mycoides* subsp. *mycoides* SC lack a substantial segment of genetic information, which must have occurred by a deletion event. We therefore conclude that the strains found in the reemerging outbreaks of CBPP in Europe during the last 15 years are derived from an ancestral strain which belonged to the African cluster and which seems to represent the original type of *M. mycoides* subsp. *mycoides* SC.

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