Original article

Antigenic and genetic characterisation of lipoprotein LppC from *Mycoplasma mycoides* subsp. *mycoides* SC

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Abstract – Lipoprotein LppC, an immunodominant antigen, and its corresponding gene *lppC* were characterised in *Mycoplasma mycoides* subspecies *mycoides* small colony (SC) type, the etiological agent of contagious bovine pleuropneumonia (CBPP). The *lppC* gene was found in the type strain of *M. mycoides* subsp. *mycoides* SC and in field strains isolated in Europe, Africa, and Australia, as well as in vaccine strains. Southern blot analysis indicated the presence of at least four copies of *lppC* in the genome of *M. mycoides* subsp. *mycoides* SC, of which only one seems to be functional. Genes homologous to *lppC* have also been detected in closely related mycoplasmas such as *M. mycoides* subsp. *mycoides* large colony (LC) type and in *M.* sp. bovine group 7. LppC is encoded as a precursor with a consensus sequence for a prokaryotic signal peptidase II. The amino acid sequence of LppC and its precursor showed similarity to both LppB (at the N-terminal domain) and LppQ (at the C-terminal domain), two lipoproteins described previously in *M. mycoides* subsp. *mycoides* SC. The N-terminal domain of the mature LppC seems to be surface exposed. The C-terminal domain presented an integral membrane structure made up of five repeated units, rich in hydrophobic and aromatic amino acids, which may have pore forming potential in the mycoplasmal membrane. A recombinant peptide representing the N-terminal half of LppC was obtained following cloning in vector pETHIS-1 and expression in *Escherichia coli* hosts. The recombinant protein was used on immunoblots for serological analysis of sera from cattle that were naturally or experimentally infected with *M. mycoides* subsp. *mycoides* SC.

***Mycoplasma mycoides* subsp. *mycoides* SC / contagious bovine pleuropneumonia / multiple copy gene / antigenic protein / lipoprotein**

1. INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is an infectious respiratory disease caused by *Mycoplasma mycoides* subspecies *mycoides* small colony (SC) type. The disease causes great economic losses in cattle production, primarily in Africa, where the disease is endemic. Due to its significant impact on animal health and livestock production, CBPP is classified as a list-A disease by the Office International des

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Epizooties (OIE), which indexes the most severe contagious animal diseases. CBPP was almost eradicated from Europe in the first half of the 20th century. However, since 1980 the disease has re-emerged in some south European countries. These re-emerging outbreaks in Europe were due to a particular cluster of *M. mycoides* subsp. *mycoides* SC strains which were not present on other continents, as observed by IS*1296* typing [7]. In this respect, small ruminants have been proposed as possible reservoirs of the pathogen [22, 25]. Currently, immunoprophylaxis is based on the use of attenuated live vaccine strains of *M. mycoides* subsp. *mycoides* SC [20]. These vaccines give relatively short post-vaccinal protective immunity and some doubts about their safety have recently arisen [13]. In order to develop new strategies in the design of vaccines against CBPP, it is essential to understand the molecular mechanisms of the pathogenicity of *M. mycoides* subsp. *mycoides* SC and to know the nature and function of its antigenic components.

Virulence factors in mycoplasmas seem to be related to structural compounds or metabolic products [26, 27]. Thus, membrane lipoproteins of several pathogenic mycoplasmas have been suggested as possible virulence factors due to their capability to induce blastogenesis and the secretion of proinflammatory cytokines in vitro [6, 11, 21].

The major antigenic proteins, as revealed on immunoblots containing total antigen of *M. mycoides* subsp. *mycoides* SC and reacted with serum from cows with CBPP, have molecular masses of 110, 98, 95 85, 80, 72, 62, 48, 44, and 39 kDa [1, 9]. These typical antigens are currently used to confirm sera that were positive in the complement fixation test (CFT) for CBPP. Currently, four lipoproteins of *M. mycoides* subsp. *mycoides* SC have been characterised. They include LppA (also known as P72), a 72 kDa membrane-located lipoprotein that is also found in *Mycoplasma* sp. bovine group 7 with only minor differences [8];

LppB, a lipoprotein of 70 kDa which is absent in new European strains of *M. mycoides* subsp. *mycoides* SC [29]; LppQ, a 48-kDa lipoprotein that appears to be highly specific for the species *M. mycoides* subsp. *mycoides* SC [2]; and Vmm, a variable surface protein of 59 amino acids that undergoes reversible phase variation [18].

In the present work, we characterised an immunodominant antigen, the putative lipoprotein LppC and its *lppC* gene in different strains of *M. mycoides* subsp. *mycoides* SC, as well as gene sequences homologous to *lppC* in other closely related mycoplasmas.

2. MATERIALS AND METHODS

2.1. 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 I. Mycoplasmal cultures were grown in a standard mycoplasma medium [4] to a density of 10^8–10^9 cells/mL. Crude lysate preparations with GES buffer (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% *N*-lauroylsarcosine) and DNA extraction were performed as previously described [7].

For genetic manipulation and subcloning, *Escherichia coli* strains XL1-Blue MRF' and XLOLR (Stratagene, La Jolla, CA, USA) were used. *E. coli* strains were grown in Luria-Bertani (LB) broth at 37 °C. The phagemid expression vector pBK-CMV (Stratagene) was propagated in the *E. coli* strain XLOLR. For subcloning, the pBluescriptII SK⁻ vector (Stratagene) was propagated in the *E. coli* XL1-Blue strain (Stratagene). The pETHIS-1 expression vector [24] was used for poly-histidine fusion at both N-terminal and C-terminal ends of cloned proteins. The *E. coli* strain BL21(DE3) (Novagen, Madison, WI, USA) was used for expression of recombinant proteins.

Table I. *Mycoplasma* strains used.

Mycoplasma species	Strain[a]	Origin	Year isolated	Host	lppC[c]	TA[e]
M. mycoides subsp. *mycoides* SC	PG1		1931	Cattle/type strain	+	8;9;11;17;18
	2059	Spain	1984	Cattle/lung	+	9
	B773/125	Portugal	1991	Cattle/semen	+	10
	C305	Portugal	1993	Goat/lung	+	10;11
	O326	Portugal	1993	Sheep/milk	+	10;11
	PO 2	France	1980	Cattle/lung	+	11;12
	2022	France	1984	Cattle/lung	+	10
	L2	Italy	1993	Cattle/lung	+	9
	402	Italy	1990	Cattle/lung	+	11;12;13
	6479	Italy	1992	Cattle/lung	+	9;10
	Afadé	Cameroon[b]	1968	Cattle/lung	+	9;10
	Fatick	Senegal	1968	Cattle	+	9;10
	B17	Chad	1967	Zebu	+	10;11
	9050–529/1	Ivory Coast	1990	Cattle	+	10
	91130	Central African Republic	1991	Cattle	+	9;10
	94111	Rwanda	1994	Cattle	+	12;13
	95014	Tanzania	1995	Cattle	+	10
	T1/44	Tanzania	1952	Cattle/vaccine strain	+	10;11
	T1/Sr50	Tanzania	1952	Cattle/vaccine strain	+	9
	Gladysdale	Australia		Cattle	+	10;11
	V5	Australia	1965–8	Cattle/vaccine strain	+	10
	R575	Australia	1965–8	Cattle	+	9
	DVZ	Australia	1965	Cattle	+	9;10
M. mycoides subsp. *mycoides* LC	Y-goat	Australia		Goat/type strain	+	
M. mycoides subsp. *capri*	PG3			Goat/type strain	+[d]	
Mycoplasma sp. bovine group 7	PG50	Australia		Cattle/reference strain	+	
M. capricolum subsp. *capricolum*	California kid	California		Goat/type strain	–	
M. capricolum subsp. *capripneumoniae*	F38	Kenya		Goat/type strain	–	
M. agalactiae	PG2	Spain	1973	Goat/type strain	–	
M. bovis	PG45			Cattle/type strain	–	
M. putrefaciens	KS1			Goat/type strain	–	

[a] Collections: the strains were obtained from the National Collection of Type Cultures (NCTC), PHLS, London, United Kingdom; Laboratoire de Pathologie Bovine, Lyon, France; Laboratório Nacional de Veterinaria, Lisbon, Portugal; CIRAD-EMVT, Montpellier, France; Institute for Veterinary Bacteriology, University of Berne, Berne, Switzerland; and Australian Animal Health Laboratory, Geelong, Victoria, Australia.
[b] Isolated at Farcha Laboratory, N'Djaména, Chad, from a bovine from Afadé, Northern Cameroon.
[c] As tested by Southern blot hybridisation with the gene probe C2; – indicates no hybridisation signal.
[d] A very faint band reacted with the gene probe C2.
[e] Number of TA repeats in the promoter area of *lppC*. Note the presence of a variable number of dinucleotide repeats within the same strain, indicating a heterogeneous mycoplasma population. Underlined numbers refer to the predominant variants for each strain.

2.2. DNA manipulation, construction and screening of an expression library

DNA of the *M. mycoides* subsp. *mycoides* SC Afadé strain was partially digested with *Sau*3AI to obtain fragments ranging from 2 to 10 kb. DNA fragments were ligated with *Bam*HI-digested λ Zap Express cloning vector (Stratagene), and packaged with Gigapack II packaging extract (Stratagene). The library was plated according to the manufacturer's protocol using the *E. coli* host strain XL1-Blue MRF'. Screening of the library was performed following a standard protocol with serum from a cow experimentally infected with the Afadé strain (cow #512, serum taken at slaughter, 42 days post-infection) [1] used at a dilution of 1:100. Positive clones were selected, amplified and excised in vivo into pBK-CMV phagemids using the filamentous helper phage ExAssist (Stratagene) as indicated by the manufacturer.

A partial genomic library of the *M. mycoides* subsp. *mycoides* SC L2 strain was made by cloning total DNA digested with *Hin*dIII into the *Hin*dIII site of the pBluescriptII SK⁻ vector. Clones were selected by colony screening with the digoxigenin-11-dUTP-labelled C2 gene probe produced by PCR using oligonucleotide primers MMMSCAFIIC1 and MMMSCAFIIC2 (Tab. II) designed against part of the *lppC* gene [19]. Plasmid DNA of selected colonies were isolated using the QIAprep™ Spin Miniprep kit (Qiagen AG, Basle, Switzerland).

2.3. Molecular biology techniques and bioinformatic analysis

PCR reactions and the Southern blot analysis were performed as described previously [19] with the specific primers listed in Table II.

Amplified *lppC* products, obtained with primers MMMSCL7 and MMMSCAFIIC7 from the 23 *M. mycoides* subsp. *mycoides* SC strains tested (Tab. I), were cloned into

the pGEM-T vector (Promega, Madison, WI, USA) and the constructs were subsequently propagated in the *E. coli* XL1-Blue strain. Plasmid DNA of six randomly selected colonies for each strain was isolated and subjected to sequencing.

DNA sequencing and analysis were performed as previously described by Abdo et al. [1]. Alignments were performed with the Wisconsin package (Genetics Computer Group, Inc. [GCG], Madison, WI, USA) and with the ClustalW 1.8 and BOXSHADE 3.21 programs both from EMBnet.ch (http://www.ch.embnet.org/index.html). Transmembrane prediction of the protein was made by using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) [12] and by the method of Kyte and Doolittle [14].

2.4. Production of poly-histidine-tailed fusion protein LppC

A poly-histidine-tailed LppC fusion protein was obtained by amplification of the N-terminal part of LppC, from amino acid 24 to amino acid 254, using genomic DNA of the Afadé strain and the primers MMMSCL1 and MMMSCL2 (Tab. II), containing a *Hin*dIII and a *Not*I restriction site overhang, respectively. The PCR product was digested by *Hin*dIII and *Not*I and cloned into pETHIS-1. The resulting plasmid pJFFLppC-His was verified by restriction enzyme digestion and partial DNA sequencing. Expression and purification of poly-histidine tailed LppC was made as described previously for lipoprotein LppB [29]. The recombinant protein had an apparent molecular mass of approximately 32 kDa (data not shown) which corresponded to the calculated mass of 31.4 kDa for LppCN'-His.

2.5. CBPP sera and production of monospecific rabbit anti-LppCN'-His antibodies

Sera taken sequentially from cattle that had undergone a controlled experimental

Table II. Oligonucleotide primers used in this study.

Name	Sequence[a]	Position[b]	Annealing temp. (°C)[c]
MMMSCAFIIC1	TGTTGGTTTAGTTGCAACAAC	1156–1176	49.5
MMMSCAFIIC2	CTCCCTAAACGTAATTTAACC	1566–1546	48.6
MMMSCAFIIC3	ACGTTTTGTTGAGTTAAAAATGG	2935–2913	50.1
MMMSCAFIIC4	TGTTCAGTTATCATCTGCAAG[d]		48.5
MMMSCAFIIC5	TTTCTGCAGACTTAGTACTTG	1232–1212	47.6
MMMSCAFIIC6	AAAAAATTCTGAGGTCATAAAAG	1357–1379	49.0
MMMSCAFIIC7	AAAACTGGCCATCTTGATCTTC	1676–1655	50.4
MMMSCAFIIC8	AAAATGTAGTTATTACTCAAATAG	1698–1721	47.1
MMMSCAFIIC10	CCACAGACCATAGTAAAAGC[d]		48.3
MMMSCAFIIC11	GCTATGCTCTATCTTATTTTTTG[d]		48.6
MMMSCAFIIC12	ACAAATGTAACCAACAATATGG[d]		49.0
MMMSCL1	GTaagcttTTGTGGTGATAAAACACCAAGT	1187–1216	49.8[e]
MMMSCL2	GAgcggccgcATTTATCTAAGTTTAAAACTTCTTT	1899–1865	47.8[e]
MMMSCL3	CTAACCCGCAAACTTCAAACC	256–276	51.0
MMMSCL4	GATCCTTTTCCGTTCTCATAATC	803–781	50.0
MMMSCL5	GCTGCTGCAACAAAGTTTAACC	2003–2024	51.1
MMMSCL6	GGAGATTCTGAATTTTGATTATTG	361–338	49.7
MMMSCL7	ATAATGCTGTTTCTCAATTAAAAG	697–720	49.1
MMMSCL8	GGTTAAAATCTGTTGCATCATAG	2099–2077	49.4
MMMSCL9	TACTGTCTTAGGTATTAATAGC	2557–2536	46.5
MMMSCL10	GGAAAAGACTTGTTTTAAAAAAC	2588–2610	49.1
MMMSCL11	AACGGAAAAGGATCTGGATCTG	790–811	51.1
MMMSCL12	TACAACCAACATATTTTTCTTTC	1754–1732	49.7

[a] Lower-case letters indicate nucleotides added to create restriction enzyme recognition sites for cloning.
[b] Based on nucleotide sequence AY026041 (3.1-kb insert of plasmid pJFFL2IA, the insert deriving from the European strain L2).
[c] 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 the target GC content and 1000 bp as the target size.
[d] Primers designed on the insert of plasmid pJFFAfIIC2.
[e] Not considering the nucleotides added to create the restriction enzyme recognition site.

infection with *M. mycoides* subsp. *mycoides* SC, the European strain L2 (cow #502) and African strain Afadé (cows #511 and #512), which have been described in detail [1]. Sera from cows that were naturally infected were obtained from F. Santini, Teramo, Italy and F. Poumarat, Lyon, France and had CFT titers ranging from 1:10 to 1:1280.

Monospecific, polyclonal antibodies directed against LppCN'-His were obtained by immunising a rabbit subcutaneously with approximately 40 μg of recombinant polyhistidine-tailed LppC in 450 μL saline buffer (50 mM NaH_2PO_4, pH 8.0, 0.85% NaCl) mixed with 450 μL Freund complete adjuvant (Difco Laboratories, Detroit, MI, USA). Two booster immunisations with

approximately the same amount of protein in Freund incomplete adjuvant (Difco Laboratories) were performed 21 days and 33 days after the first injection. The animal was bled 16 days after the second booster immunisation, according to standard protocols [10] and rabbit antiserum against LppCN'-His was collected.

2.6. Immunoblot analysis and Triton X-114 partitioning

Proteins were separated by 10 or 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [15] and transferred onto nitrocellulose membranes (BioRad Laboratories, Hercules, CA, USA) according to standard protocols [3]. For immunoblotting, Western blots were blocked with 1% milk buffer for at least one hour and then incubated with the rabbit antiserum against LppCN'-His (1:1000) or different CBPP sera (1:100) in milk buffer overnight at 4 °C on a shaker. The membranes were then washed thoroughly with deionised water before incubation with alkaline phosphatase-labelled conjugates goat anti-rabbit IgG (H+L) (Kirkegaard & Perry, Gaithersburg, MD, USA) or monoclonal anti-bovine IgG (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:2000 and 1:5000 in 1% milk buffer, respectively, for 90 min at room temperature. The colour reaction was initiated by the addition of nitroblue tetrazolium and bromochloroindolyl phosphate in alkaline substrate buffer (7 mM Na_2CO_3, 3 mM $NaHCO_3$, pH 9.6, 1 mM $MgCl_2$).

Immunostaining on colony plaque fingerprints was performed by a modification of the method described by Persson et al. [18]. Briefly, between 10 000 and 50 000 colony plaques of *M. mycoides* subsp. *mycoides* SC strains PG1, O326, L2, Afadé and T1/Sr50 were grown on agar plates and transferred onto nitrocellulose membranes by placing the membranes on the surface of agar plates. The membranes were gently removed and blocked with 1% milk buffer prior to immunoblot-

ting with the monospecific anti-LppCN' serum (1:1000).

M. mycoides subsp. *mycoides* SC total antigen was separated into hydrophobic and hydrophilic fractions by the Triton X-114 partitioning method [5] using a 10-mL culture of Afadé grown until the stationary phase. Samples from the Triton X-114 detergent phase, the aqueous phase, and whole mycoplasma cells were mixed with protein sample buffer, run on SDS-acrylamide gels, and blotted onto nitrocellulose. The filter was subsequently used for immunoblotting with the monospecific, polyclonal antibodies directed against LppC.

2.7. Nucleotide sequence accession numbers

The EMBL/GenBank accession number of the cloned fragment containing the *lppC* gene of plasmid pJFFL2IA from *M. mycoides* subsp. *mycoides* SC L2 strain is AY026041. Sequence data of the *lppC* gene of plasmid pJFFAfIIC2 from Afadé are accessible under AY026042.

3. RESULTS

3.1. Cloning and sequence analysis of *lppC* from *M. mycoides* subsp. *mycoides* SC

An expression library based on the bacteriophage vector Zap Express was obtained from genomic DNA of the *M. mycoides* subsp. *mycoides* SC Afadé strain. Phage clones were screened using serum from a cow experimentally infected with *M. mycoides* subsp. *mycoides* SC. One clone out of 5 000, named pJFFAfIIC2 (Fig. 1), reacted strongly with the CBPP serum and was selected for DNA sequence analysis. It contained a 4.2 kb insert and revealed the presence of part of a gene encoding a potential lipoprotein named LppC; the gene was designated *lppC*. PCR analysis with primers MMMSCAFIIC1/MMMSCAFIIC3
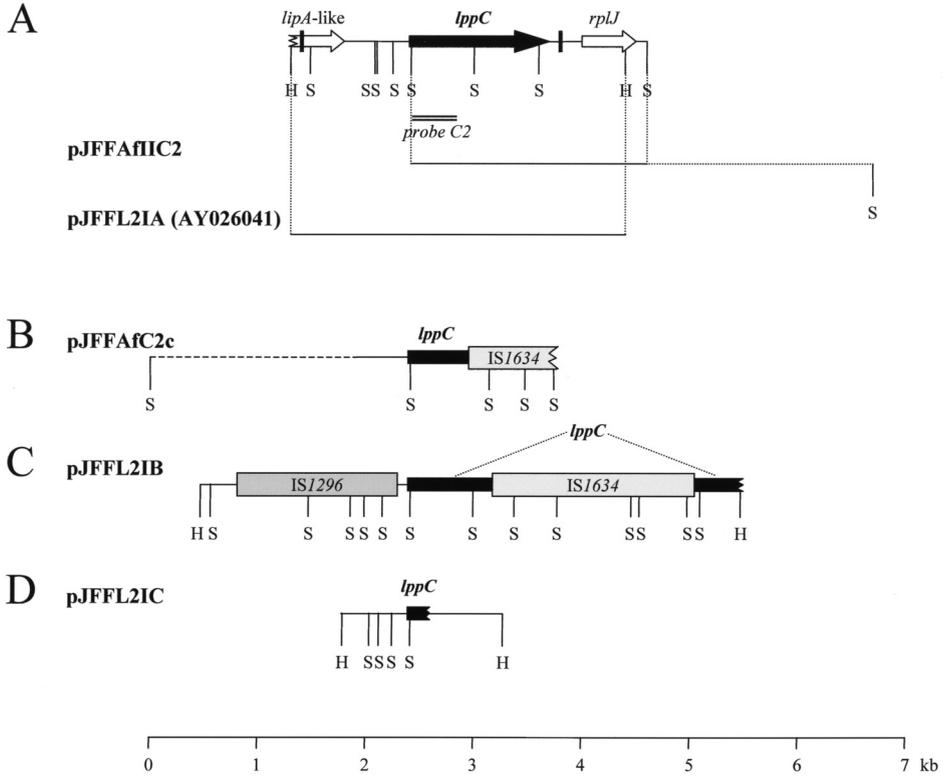
Figure 1. Plasmids investigated in this study. The four *lppC* genes found are the complete, functional copy (*A*); the IS*1634*-disrupted copies (*B* and *C*); and the truncated form (*D*). Clones pJFFL2IA, pJFFL2IB and pJFFL2IC were derived from a plasmid bank created with *Hin*dIII-digested genomic DNA from the *M. mycoides* subsp. *mycoides* SC strain L2. Both clones pJFFAfIIC2 and pJFFAfC2c derive from a phage bank created with *Sau*3AI-digested genomic DNA of the Afadé strain. H, the cut site of *Hin*dIII; and S, the cut site of *Sau*3AI. Vertical black bars represent the 85-bp direct repeats. The dotted line indicates the non-contiguous half of pJFFAfIIC2 and the interrupted line indicates the non-sequenced portion of pJFFAfC2c. The localisation of the gene probe C2 is indicated. The genetic maps are aligned with the 5' end of the functional *lppC* (plasmid pJFFL2IA).

and MMMSCAFIIC1/MMMSCAFIIC4 (Tab. II) revealed the integrity of the clone. The remaining part of *lppC* was then obtained using PCR technology. By screening the expression library with the specific C2 probe (Fig. 1) plasmid pJFFAfC2c was obtained and was found to contain part of another copy of *lppC* that was interrupted by the insertion sequence IS*1634* (Fig. 1).

To investigate the presence of *lppC* genes in a re-emerging European strain of *M. mycoides* subsp. *mycoides* SC, a plasmid gene bank was produced with *Hin*dIII digested genomic DNA of L2 strain and screened with the C2 probe. Three positive clones, plasmids pJFFL2IA, pJFFL2IB, and pJFFL2IC, were selected (Fig. 1). Sequence analysis revealed that the pJFFL2IA insert

consisted of a contiguous segment that corresponded exactly to the DNA segment carried by pJFFAfIIC2 (Fig. 1). It contained an open reading frame (ORF) of 1 236 bp, encoding a protein of 411 amino acids with a calculated molecular mass of 47.3 kDa. The coding sequence of *lppC* contains 7 mycoplasma-specific TGA_{Trp} codons.

The *lppC* ORF is preceded by a consensus sequence for a putative ribosomal binding site located 5 nucleotides upstream of the start codon, a possible −10 box (TATAATG) located 43 nucleotides upstream of the start codon, and a possible −35 box (TTGACA) located another 16 nucleotides upstream of the −10 box. No significant stem loop structure was found downstream of the stop codon. Further downstream, we identified the *rplJ* gene encoding the ribosomal 50S subunit protein L10. Upstream of the *lppC* gene, we detected the 3'-terminal part of a putative gene resembling the *lipA* gene of *M. pulmonis* and a stem loop structure with a ΔG of −5.8 kcal/mol representing a potential transcription stop signal.

Two 85-bp direct repeats were detected on plasmid pJFFL2IA (Fig. 1). The first one was located in the *lipA*-like ORF upstream of *lppC* beginning at base 47 and ending at base 131 of the 3337-bp sequence. The second one was found in the non-coding region downstream of the *lppC* gene beginning at base 2387 and ending at base 2471. The sequences of the 40 bases flanking both repeats on their 5' ends were very similar. The 35 bases forming the 3' ends of these two direct repeats were identical to a portion of IS*1296*, with the exception of one nucleotide substitution.

Even though the coding regions of *lppC* in *M. mycoides* subsp. *mycoides* SC strains Afadé and L2 were identical, two differences were found on the segments flanking *lppC*. A poly-G region, beginning 104 nucleotides downstream of the *lppC* gene contains eight G's in L2 strain and only seven in the Afadé strain. The other difference found in the L2 strain compared to the Afadé strain was located in the promoter

area 46 nucleotides upstream of the start codon of the *lppC* gene. There, a polymorphism was found in a poly-TA sequence flanked by two inverted repeats (ACAT and ATGT). This palindromic sequence included the last three bases of the possible −35 box and the whole putative −10 box of the gene *lppC* and formed a stem loop structure with a ΔG of −9.6 kcal/mol. The L2 strain possesses nine TA dinucleotides while genomic DNA of the Afadé strain has principally ten. PCR amplification with primers MMMSCL7 and MMMSCAFIIC7 (Tab. II) for all strains followed by cloning and sequencing with primers MMMSCL11 and MMMSCAFIIC2 (Tab. II) revealed that the number of TA repeats in this locus varied from 8 to 18 copies (Tab. I). Moreover, DNA sequencing of these cloned fragments containing the *lppC* promoters of given strains revealed that the number of TA repeats varies within almost each strain, indicating the occurrence of mycoplasma subpopulations with a polymorphism in the *lppC* promoter region.

3.2. Analysis of the amino acid sequence of the lipoprotein LppC

Sequence analysis of the amino acid sequence deduced from the *lppC* gene revealed significant similarity with the hypothetical surface-located membrane protein LppB of *M. mycoides* subsp. *mycoides* SC (EMBL/GenBank accession number AF165135), showing 37% identity and 49% similarity, and with the membrane associated lipoprotein precursor LppQ of *M. mycoides* subsp. *mycoides* SC (EMBL-GenBank accession number AF072716), showing 31% identity and 47% similarity. Analysis of the amino acid sequence of LppC revealed a typical prokaryotic signal peptidase II cleavage site in the N-terminal region containing two Lys residues in the first seven amino acids. The peptidase cleavage site consists in Val-Ile-Ala_Cys-Gly, the cystein residue being at position 24. Hence, the resulting mature LppC protein of 388 amino acids had a calculated
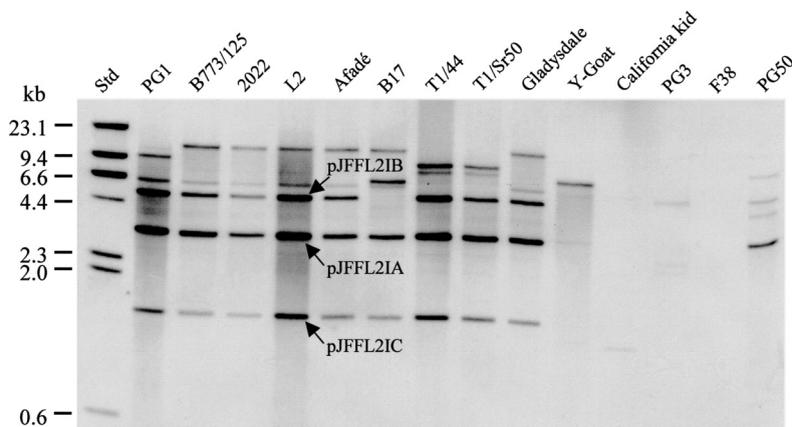
Figure 2. Detection of individual copies of *lppC* in a selection of strains of *M. mycoides* subsp. *mycoides* SC and other mycoplasma species by Southern hybridisation. Three hundred nanograms of genomic DNA were digested with *Hin*dIII, separated electrophoretically on 0.7% agarose gels, transferred onto positively charged nylon membranes, and hybridised to the DIG-labelled *lppC* C2 probe. The bands corresponding to full or partial *lppC* genes as cloned in pJFFL2IA, pJFFL2IB and pJFFL2IC are indicated by arrows. Std, molecular mass standards (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb).

molecular mass of 45.1 kDa. The leader sequence showed a typical transmembrane helix structure, where TMpred identified a unique and significant transmembrane region (score 1814) spanning amino acids 4 to 22 in the N-terminal domain of the protein. Hydrophilicity analysis of the amino acid sequence of the non-cleaved LppC revealed that the first 25 amino acids of the N-terminal end were the most hydrophobic part of the molecule. The region following this part until amino acid 80 was particularly hydrophilic while the rest of the protein was slightly hydrophilic with little hydrophobic regions. Moreover, the C-terminal domain of LppC was shown to be built up of five repeated units composed of 25 amino acids, rich in hydrophobic and aromatic residues (Trp-X[4]-[Val/Ile]-X[2]-[Met/Leu]-X[2]-Met-Phe-X[5]-Phe-Asn-X[2]-[Ile/Leu]-X[2]) with a substitution of the twelfth amino acid (Met) by a Leu residue in the second repeat and a substitution of the twentieth amino acid (Asn) also by a Leu residue in the fifth repeat.

These repeats also supported an integral membrane location of the C-terminal part of LppC.

3.3. Evidence for multiple copies of the *lppC* gene in the *M. mycoides* subsp. *mycoides* SC genome

Sequence analysis of the *lppC* gene and the flanking genes of the individual clones from the Afadé and L2 strains revealed significant differences to the *lppC* gene on plasmids pJFFL2IA and pJFFAfIIC2 (Fig. 2), indicating three additional but incomplete *lppC* genes. One copy of the *lppC* gene, found on plasmid pJFFAfC2c, had only a few base changes in 475 nucleotides of the 5'-terminal part, but the 3'-terminal part of the gene was completely missing. In this case, a copy of the insertion element IS*1634* was found downstream of position 475 of the *lppC* gene (Fig. 1). A second copy, represented by plasmid pJFFL2IB, had an incomplete *lppC* gene that was interrupted by another copy of
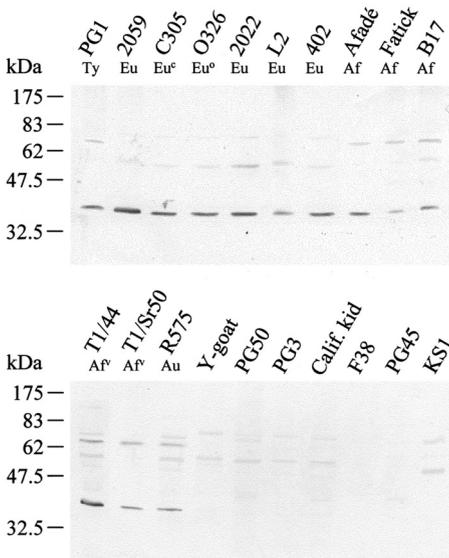
Figure 3. LppC expression in *M. mycoides* subsp. *mycoides* SC and related mycoplasmas. Immunoblot analysis was carried out with 10 µg of total antigen in each lane. Total antigen of all mycoplasmas tested was separated by SDS/12% acrylamide gels, transferred onto nitrocellulose membranes, and probed with the antiserum against LppC.

IS*1634* at position 786 (Fig. 1). Finally, a third copy, represented by plasmid pJFFL2IC, contained only a small part (approximately 190 nucleotides) of the 5' domain of *lppC* (Fig. 1). These observations led to the conclusion that *lppC* is present in more than one copy in the genome of *M. mycoides* subsp. *mycoides* SC. To confirm this, Southern blot analysis was done on genomic DNA from all *M. mycoides* subsp. *mycoides* SC strains and related mycoplasmas digested with *Hin*dIII. Detection of the *lppC* gene was performed with the gene probe C2 (Fig. 1, Tab. I). All *M. mycoides* subsp. *mycoides* SC strains tested showed the expected reacting band for the functional *lppC* gene at 3.1 kb, corresponding to the part cloned in plasmid pJFFL2IA, as well as a band at 1.5 kb which corresponded to the partial

lppC gene on the *Hin*dIII fragment in plasmid pJFFL2IC (Fig. 2). Moreover, almost all strains presented a band at 5.0 kb, corresponding to the partial *lppC* gene in plasmid pJFFL2IB (Fig. 2). Only one African strain (B17), isolated from a zebu, had a singular hybridisation pattern showing a band at 7.0 kb instead of 5.0 kb. The C2 probe reacted faintly with two other bands of all *M. mycoides* subsp. *mycoides* SC strains. One of these *Hin*dIII fragments was thought to correlate with the IS*1634*-truncated *lppC* copy found in plasmid pJFFAfC2c. The fifth fragment might be related to another unknown truncated form of *lppC* or to a similar lipoprotein gene. The C2 probe also showed a weak hybridisation signal with DNA from the *M. mycoides* subsp. *mycoides* LC type strain Y-goat. The *M.* sp. bovine group 7 reference strain PG50 showed a strongly reacting band at 3.1 kb which harbours an *lppC*-like gene. This *lppC* homologue presented 83% identity in 577 nucleotides of the 5' end with the *lppC* gene of plasmid pJFFL2IA. At the amino acid level, this corresponded to 73.5% identity and 87% similarity in a portion of 189 amino acids.

3.4. Expression of LppC in *M. mycoides* subsp. *mycoides* SC

All *M. mycoides* subsp. *mycoides* SC strains reacted with anti-LppCN'-His antibodies on immunoblots revealing a distinct band at 39 kDa (Fig. 3). In addition, the blots also revealed a band at 70 kDa cross-reacting with anti-LppCN'-His in all strains except in those from Europe. The other mycoplasma species did not react with anti-LppCN'-His antibodies (Fig. 3). In a control experiment using the same antigen preparations, we observed expression of LppQ in all *M. mycoides* subsp. *mycoides* SC strains tested. Analysis of immunostained colonies of *M. mycoides* subsp. *mycoides* SC strains using anti-LppC serum did not reveal any non-stained sectors, indicating that LppC is not a variable antigen.
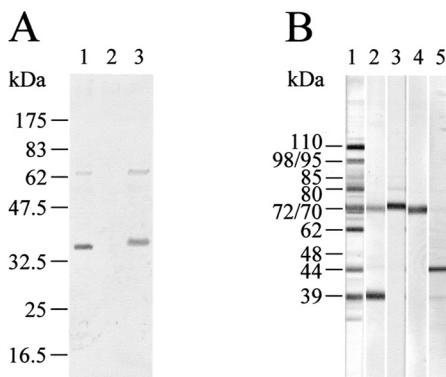
Figure 4. Triton X-114 phase partitioning of the *M. mycoides* subsp. *mycoides* SC Afadé strain. (*A*) Mycoplasmas at the end of exponential growth were submitted to Triton X-114 partitioning; 15 μL of fractions and total antigen were separated by SDS/12% acrylamide gels, transferred onto a nitrocellulose filter, and probed with monospecific LppC antiserum. Lane 1, total antigen; lane 2, aqueous phase; lane 3, detergent phase. (*B*) Western blot analysis of Triton X-114 extract from Afadé using antiserum from cow #512 at day 35 (CFT titer of 1:10240) post-infection with the Afadé strain (lane 1) and monospecific rabbit antibodies against LppC (lane 2), LppA (P72) (lane 3), LppB (lane 4) and LppQ (lane 5).

3.5. Identification of LppC as a membrane protein

To determine the cellular location of LppC, total antigen of the *M. mycoides* subsp. *mycoides* SC Afadé strain was submitted to Triton X-114 phase partitioning. Monospecific anti-LppC serum reacted strongly with a 39-kDa protein and weakly with a 70-kDa protein in the Triton X-114 phase but not with any proteins found in the aqueous phase (Fig. 4A). This indicates that LppC is an integral membrane protein. This corresponds to calculations based on the amino acid sequence of LppC, as mentioned before. Western blot analysis of the Triton X-114 extract with serum from a cow experimentally infected with the Afadé strain showed one band of approximately 39 kDa corresponding to LppC

(Fig. 4B). Immunoblots with the monospecific antibodies against LppA (P72), LppB and LppQ, which were used as controls, revealed similar reaction strengths as found with the anti-LppCN' serum. Furthermore, anti-LppB antibodies revealed a band at 70 kDa that migrated to the same position on the gel as the 70-kDa band reacting with the anti-LppCN' antibodies.

3.6. Immunogenicity of LppC

Membrane strips containing the recombinant LppCN'-His protein were reacted with sera from cattle, taken sequentially before and after experimental contact infection with *M. mycoides* subsp. *mycoides* SC strains L2 (cows #502) and Afadé (cow #511), respectively. The infection of these two cows was performed by housing them in contact with inoculated animals. Serum from cow #502 gave a weak signal at 126 days post-infection. This signal persisted albeit at lower levels until 224 days of infection (Fig. 5A). In the case of cow #511, the first serum showing a positive reaction to LppCN'-His was the one collected 49 days post-infection. Strong signals could be detected with sera collected between days 56–98 post-infection. Afterwards, the signals weakened but were still easily detectable until the serum taken at slaughter 147 days post-infection (Fig. 5B). Analysis of sera from naturally infected European cattle gave strong serological reactions with recombinant LppCN'-His, except for a weakly positive serum (gamma) with a CFT titer of 1:10 (Fig. 5C).

4. DISCUSSION

Western blot analysis using sera from cattle with CBPP revealed proteins with the molecular masses of 110, 98, 95, 85, 80, 72, 62, 48, 44, and 39 kDa as the most common antigenic bands among all *M. mycoides* subsp. *mycoides* SC strains [1, 9]. The group constituting African and Australian strains and the type strain PG1 was also
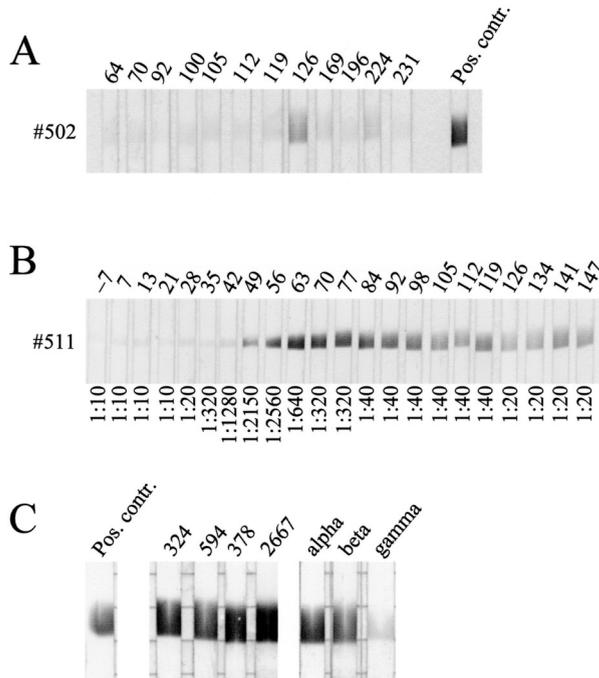
Figure 5. Appearance of antibodies against LppC during experimental and natural infections with *M. mycoides* subsp. *mycoides* SC. The immunoblots contain purified recombinant LppCN'-His and were reacted with cow sera taken sequentially before and after experimental infection with the L2 and Afadé strains, or with European CBPP sera. (*A*) LppCN'-His reacted with serum from cow #502 contact infected with L2. The numbers on the top of each row indicate days after infection. (*B*) LppCN'-His reacted with serum from cow #511 contact infected with Afadé. The CFT titers for sera from cow #511 are given at the bottom of each row. The numbers on the top of each row indicate days before (-) or after infection. (*C*) LppCN'-His reacted with seven CBPP sera from France and Italy. Sera 324, 594, 378 and 2667 had CFT titers of 1:1280. Serum alpha had a titer of 1:1280, serum beta was at 1:320 and serum gamma at 1:10. Pos. contr., serum from cow #511 at day 70 (CFT titer of 1:320) post-infection.

characterised by the presence of a 70 kDa antigen, corresponding to lipoprotein LppB, which was not detected in European strains [9, 29]. In addition, two other antigenic proteins have been characterised thus far in *M. mycoides* subsp. *mycoides* SC: the 72 kDa antigen, also known as LppA [8, 16, 17] and the 48-kDa protein LppQ [2]. In an attempt to characterise the other major antigenic protein bands, we identified the *lppC* gene. This gene encodes an immunogenic lipoprotein of *M. mycoides* subsp. *mycoides* SC corresponding to the antigenic band at

39 kDa. The deduced amino acid sequence of LppC showed similarities with the two other known lipoproteins of *M. mycoides* subsp. *mycoides* SC, LppB [29] and LppQ [2]. This could explain why antibodies against the N-terminal domain of LppC cross-react slightly with LppB which is present in the type strain and in African and Australian strains. The C-terminal parts of LppC and LppQ were also similar, consisting of five repeated units, rich in hydrophobic and aromatic amino acids in LppC, or nine of the same repeats in LppQ [2]. In

analogy to LppQ, this structure found in LppC gives theoretical evidence for an integral membrane location [23]. The repeated amino acid motif might be involved in pore formation in the mycoplasmal membrane, building a channel for passive transport (inward and/or outward) of molecules through the membrane of *M. mycoides* subsp. *mycoides* SC. The LppC protein was shown to be membrane-located by Triton X-114 partitioning experiments and to harbour a single transmembrane region corresponding to the signal sequence. These data, together with the finding that recombinant LppC reacted strongly with the serum of cattle infected with *M. mycoides* subsp. *mycoides* SC, suggest that this lipoprotein is an outer membrane protein.

Southern blot and sequence analysis gave evidence for the presence of one complete copy of *lppC* and a further three partial copies of the same gene in the genome of *M. mycoides* subsp. *mycoides* SC. Two copies were interrupted by the insertion sequence IS*1634* [28]. Most of the field strains tested, originating from Europe as well as from Africa and Australia, showed the same hybridisation profiles indicating that the different *lppC* genes are on the same genetic loci in both African as well as European strains. Only one African field strain isolated from a zebu, the two vaccine strains T1/44 and T1/Sr50, and the type strain presented unique patterns that may be explained by differences at the enzymatic cutting sites. *Mycoplasma* sp. bovine group 7 reference strain PG50 and the *M. mycoides* subsp. *mycoides* LC type strain Y-goat, both members of the *M. mycoides* cluster and closely related to *M. mycoides* subsp. *mycoides* SC, also revealed genes homologous to *lppC*.

Western blot analysis performed with anti-LppC serum and on whole cell antigen of several *M. mycoides* subsp. *mycoides* SC strains revealed a major band at 39 kDa in all strains analysed as well as a minor band at 70 kDa in non-European strains. This indicates that the mature LppC protein

has an apparent molecular mass of 39 kDa on SDS-PAGE. This value is significantly lower than the molecular mass calculated from the deduced amino acid sequence of the mature protein (45.1 kDa), suggesting that LppC migrates abnormally on SDS-PAGE. Such anomalous migration of mycoplasmal membrane proteins has been reported previously [8, 30]. On the basis of the amino acid sequence, the most plausible explanation for this anomalous migration may be the potential for an unusual or denaturation-resistant protein conformation imparted by the particularly hydrophilic domain with charged residues. In this regard, it has to be noted that the calculated pI of the protein is 8.7. During an experimental infection, the cow infected with the African strain Afadé developed antibodies reacting with the recombinant protein LppCN'-His seven weeks postinfection, whereas the cow experimentally infected with the European strain L2 did not show any significant reaction until the time of slaughter. On the contrary, six sera from European cows that naturally acquired CBPP reacted strongly with LppCN'-His. The rather low serological response to LppC in an animal experimentally infected with the L2 strain reflects the generally low serological response of this animal to *M. mycoides* subsp. *mycoides* SC [1].

Genetic analysis revealed a TA repeat polymorphism upstream of the *lppC* gene for several *M. mycoides* subsp. *mycoides* SC strains. This poly-TA locus is part of a palindromic sequence forming a hairpin structure and includes three of six bases within the potential −35 box and the entire putative −10 box of the *lppC* gene. The different strains of *M. mycoides* subsp. *mycoides* SC revealed 8 to 18 TA repeats. It is interesting to note that these TA repeats are conserved although such a structure would be assumed to be lost, unless it is of functional importance, probably in the regulation of transcription. The possible −35 box found upstream of the *lppC* gene presented a typical highly conserved prokaryotic sequence (TTGACA). The

putative −10 box is also typical for prokaryotic organisms (TATAATG). Lipoprotein Vmm has recently been reported to undergo reversible phase variation, i.e., a reversible ON/OFF switch of expression [18]. DNA sequencing of the *vmm* gene promoter from ON and OFF clones showed that the expression of Vmm is in fact regulated at the transcriptional level by TA insertions or deletions between the −35 and the −10 boxes. Clones that express Vmm and thus have an active promoter have been found to have ten such TA repeats. Any other number of repeats has been found to disrupt the functionality of the promoter [18]. In our case, however, all *M. mycoides* subsp. *mycoides* SC strains tested expressed LppC and showed no sectoring on immunostained colonies, indicating it as a stable antigen.

In conclusion, the *lppC* gene appears to encode an important, stable antigenic lipoprotein of *M. mycoides* subsp. *mycoides* SC. The gene might have been multiplied and modified during evolution, reflecting the plasticity of mycoplasmal genomes.

ACKNOWLEDGEMENTS

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