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FEMS Microbiology Letters 173 (1999) 103–110

FEMS MICROBIOLOGY LETTERS

Vsp antigens and *vsp*-related DNA sequences in field isolates of *Mycoplasma bovis*

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Received 29 September 1998; received in revised form 13 January 1999; accepted 26 January 1999

Abstract

The expression of the 1E5 epitope which is common to the three characterized variable lipoproteins VspA, VspB and VspC of *Mycoplasma bovis* type strain PG45 and the presence of *vsp* gene DNA sequences were assessed in field isolates randomly collected from cattle showing clinical manifestations due to *M. bovis* infection. Among 250 isolates tested, only four failed to react with mAb 1E5. Southern blot analysis of these four isolates and of 20 isolates expressing the 1E5 epitope were performed using synthetic oligonucleotide probes corresponding to a sequence located in the Vsp signal peptide coding region common to all known Vsp products or to selected regions of previously characterized *vsp* genes, *vspA*, *vspE* and *vspF*. The results demonstrate the presence of multiple *vsp*-related DNA sequences in all *M. bovis* field isolates tested and indicate that the *vsp* repertoire varies in size and composition among isolates.

Keywords: Genetic variation; Antigenic variation; *Mycoplasma bovis*; Gene family; Variable protein

1. Introduction

Mycoplasma bovis is one of the most pathogenic bovine mycoplasmas. This wall-less bacterium is a major causative agent of bovine mastitis, pneumonia and arthritis. It has also been associated with skin abscesses, meningitis, otitis and infections of the genital tract [1–3]. Various clinical manifestations and the isolation of the organism from multiple body sites reflect the ability of *M. bovis* to disseminate systemically after translocating through the mucosal barriers [1,4]. As many other *Mycoplasma* species it often establishes chronic infections. Due to the lack of an effective antimicrobial therapy or vaccines, *M. bovis* infections present a continuous significant problem resulting in extensive economic losses for the dairy and meat industry, in particular in Europe and North America, where the disease occurs with a high incidence [1,5]. Failure to prevent and control *M. bovis* infections is also due to the absence of specific and sensitive commercially available diagnostic tools. Previous work has focused on the identification of *M. bovis* surface components that belong to

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0378-1097/99/$20.00
PII: S0378-1097(99)00046-4

a family of multiple variable membrane lipoproteins, designated as Vsps, which are displayed at the surface of the mycoplasma cell and are prominent targets of the bovine humoral immune response [6,7]. In the type strain PG45, eight open reading frames (ORF) were identified within an 11 kbp genomic DNA fragment that present similar features and include the three characterized *vsp* genes, *vspA*, *vspB* and *vspC* [8,9]. These three *vsp* genes were shown to encode products that (i) vary in size and expression in a non-coordinate manner from one cell to its progeny [7], (ii) contain cross-reactive epitopes, i.e. epitopes that can be recognized by cross-reacting monoclonal (mAb) or polyclonal antibodies [6,7], (iii) are possibly involved in adhesion to host cells [9,10] and (iv) are among predominant antigens recognized during early and late stages of the disease [6]. Recent data suggest that the Vsps may have a diagnostic and immunoprophylactic value despite their variation (Le Grand, D., Citti, C., Bezille, P., Rosengarten, R., and Poumarat, F., unpublished results). However, a prerequisite for the development of diagnostic tools based on Vsp antigens is to define the extent of the *vsp* repertoire in terms of a sequence reservoir and the Vsp-related antigens in *M. bovis* field isolates.

In the present study we have investigated whether *vsp*-related DNA sequences also occur in *M. bovis* field isolates and whether these isolates express the Vsp epitope recognized by the 1E5 monoclonal antibody (mAb) that is present in the three characterized Vsps. To address this issue, 250 *M. bovis* isolates were randomly collected from geographically distant herds showing *M. bovis* clinical manifestations and were tested for their capacity to express the 1E5 epitope. The presence of *vsp*-related DNA sequences in *M. bovis* field isolates that do or do not express the 1E5 epitope was explored by Southern blot analysis using synthetic oligonucleotide probes representing selected regions common to all *vsp* genes or specific of well defined *vsp* genes.

2. Materials and Methods

2.1. Mycoplasma strains and isolates

M. bovis clonal variants that express defined Vsp phenotypes have been previously described and were derived from the type strain PG45 [7]. *M. bovis* field isolates were collected between 1985 and 1996 from natural outbreaks in herds showing clinical manifestations (pneumonia, mastitis or arthritis) due to *M. bovis* infection. Identification of the isolates as species of *M. bovis* was performed by dot immunobinding on membrane filtration (MFdot) [11] using species specific rabbit hyperimmune sera directed against *M. bovis, Mycoplasma bovirhinis*, *Mycoplasma bovigenitalium*, *Mycoplasma arginini, Mycoplasma agalactiae, Mycoplasma mycoides* subsp. *mycoides* LC and SC, *Mycoplasma sp.* 2D and *Acholeplasma laidlawii*. Isolates that only reacted with *M. bovis* antisera were selected. Confirmation of these isolates as species of *M. bovis* was achieved using two PCR-based assays. In the first one, PCR products were generated from each isolate using the primers *MboF*/*MboR* that amplify a 16S rDNA sequence specific for *M. bovis* and *M. agalactiae* [12]. The second method is based on the amplification of the *uvrC* gene using primers that discriminate between *M. bovis* and *M. agalactiae* [13]. The resulting collection used in this study is composed of 250 field isolates each representing a distinct herd from diverse European countries (113 isolates from France, 96 from Italy, 19 from Switzerland, 15 from Spain and seven from Germany). For the genetic analysis, each isolate was cloned three times according to the method described by Tully et al. [14]. All *M. bovis* isolates were propagated as described elsewhere [11].

2.2. Antibodies and immunobinding assays

The mAb 1E5 used in this study is an immunoglobulin (Ig) M isotype that recognizes a surface-exposed epitope on VspA, VspB and VspC [6]. The rabbit antiserum raised against *M. bovis* strain PG45 and used to pre-characterize isolates as *M. bovis* has been shown to detect VspA, VspB and VspC, but also PG45 variants in which none of these three products is expressed. Immunobinding assays performed in this study have been previously described and include dot blot immunobinding (DB) [11], Western blotting (WB) [15] and colony immunoblotting (CB) [16,17].

2.3. Oligonucleotide probes and Southern blot hybridization

Based on data previously published [8], five *vsp*-specific oligonucleotides were synthesized and used as probes in this study: S (5′-AATTCCCTTTG-TAGCAGCT-3′), RA1 (5′-CACCTGGCGAAAAT-AAAACACC-3′), RA4 (5′-CAAGGTGCAGGAA-CTAAACC-3′), RE (5′-GCACCACAACAAGGT-ACAGG-3′) and RF (5′-CCTTTAGGTGTTTC-AGGTCC-3′). Oligonucleotides were DIG-labelled using the GENSET labelling kit according to the manufacturer's recommendations (GENSET, Paris, France).

M. bovis chromosomal DNA was digested to completion with the restriction enzyme *Hin*dIII and the restricted fragments were separated by electrophoresis on 0.8% agarose gels. Southern blots and hybridization experiments [8] were carried out with a temperature of hybridization corresponding to the T_m of the oligonucleotide minus 5°C. Stringent washes were performed at the hybridization temperature in 0.2 SSC containing 0.1% of SDS (w/v) and hybridization of the probe was detected using the DIG nucleic acid detection kit according to the manufacturer (Boehringer Mannheim, Mannheim, Germany). The efficiency of genomic DNA digestion by *Hin*dIII and the efficiency of the DNA transfer onto the membrane were assessed by hybridization using a DIG-labelled probe generated by PCR with the primers *MboF*/*MboR* that amplify a region of the 16S RNA [12].

3. Results

3.1. Expression of the 1E5 target epitope among M. bovis field isolates

Expression of the mAb 1E5 target epitope was assessed in the 250 European field isolates as indicated in Section 2, using three different immunobinding assays: (i) DB, (ii) WB and (iii) CB. Based on the results (Table 1), the 250 isolates could be divided into four groups. Group 1 is composed of 230 isolates, all reacting with the mAb 1E5 in the DB assay. Group 2 is composed of 10 isolates which were negative in the DB assay but reacted with the mAb 1E5 in the WB assay. Group 3 contains six isolates in which expression of the target epitope could only be detected by CB that revealed a few positive and sectored colonies. Finally, group 4 is composed of four isolates that did not show mAb 1E5 immunobinding, regardless of the assay. These data indicate

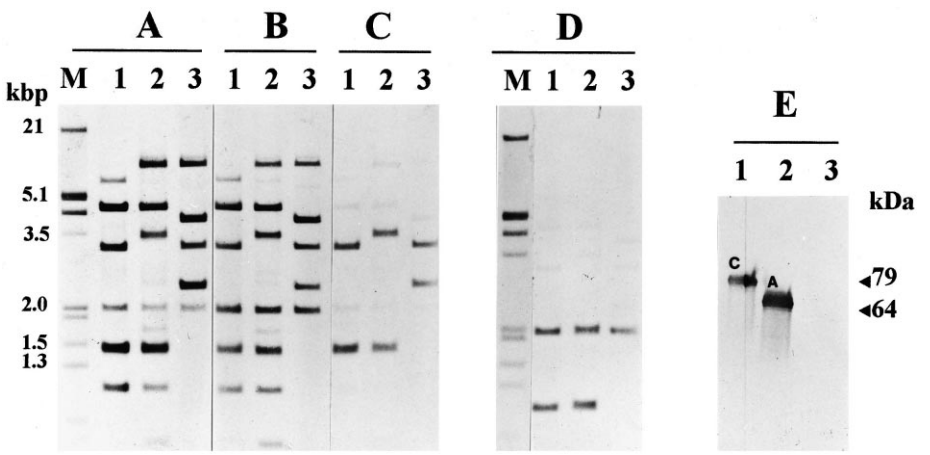

Fig. 1. (A–D) Distribution of *vsp*-associated *Hin*dIII chromosomal DNA fragments in *M. bovis* clonal isolates derived from the type strain PG45. Chromosomal DNA extracted from *M. bovis* clonal isolates (lanes 1–3) was digested to completion with *Hin*dIII and hybridized with oligonucleotide probes S (A), RA1 (B), RA4 (C) or a combination of RE and RF (D). (E) WB of whole cell lysates prepared from clonal variants depicted in A–D, lane 1–3, respectively, immunostained with mAb 1E5. Lane 1, clonal variant expressing VspC 79 kDa; lane 2, clonal variant expressing VspA 64 kDa; lane 3, clonal variant expressing neither VspA, VspB or VspC. M, Molecular size marker in kpb. The molecular mass of VspC and VspA is indicated in kDa.

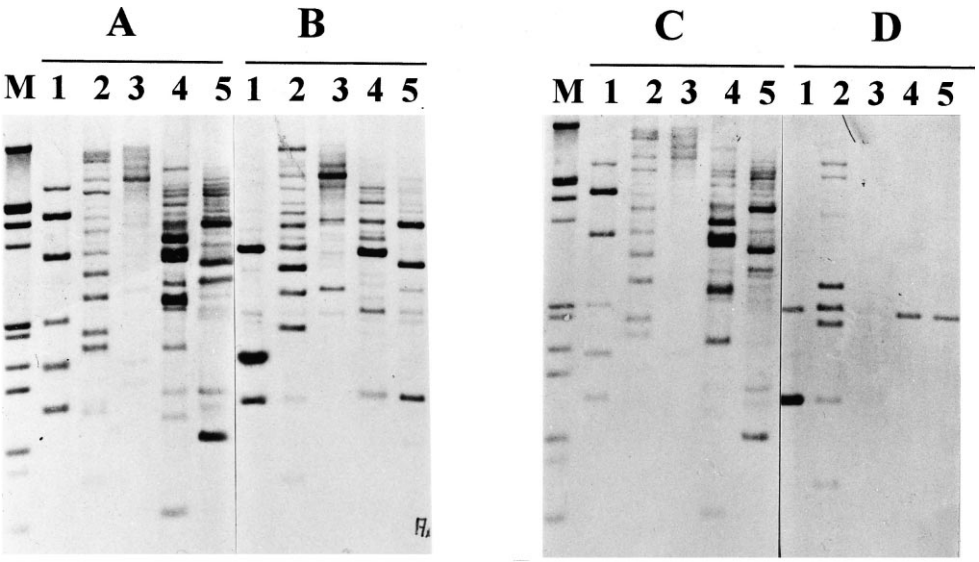

Fig. 2. Presence of *vsp*-associated *Hin*dIII DNA fragments in *M. bovis* field isolates of group 1. Chromosomal DNA extracted from a clonal isolate (lane 1, also depicted in Fig. 1, lane 1) and four *M. bovis* field isolates (lanes 2–5) randomly selected from group 1 (Table 1), was digested to completion with *Hin*dIII and hybridized with oligonucleotide probes S (A), RA4 (B), RA1 (C) or a combination of RE and RF (D). M, Molecular size marker in kpb depicted in Fig. 1.

that 98.5% of the isolates examined in this study express the 1E5 epitope suggesting the presence of antigenically related Vsp products in these isolates. The total absence of reactivity observed with the isolates of group 4 suggests that the DNA sequence encoding the target epitope is lacking in these isolates. No correlation was observed between the geographical origin of the 250 isolates and their reactivity with mAb 1E5.

3.2. *Distribution of vsp sequences within field isolates of M. bovis*

The distribution of *vsp* sequences in *M. bovis* field isolates was assessed by Southern blot hybridization of *M. bovis* chromosomal DNA digested by *Hin*dIII with a set of *vsp*-specific oligonucleotide probes that correspond to sequences (i) localized in the region encoding the signal peptide common to all Vsps, S, (ii) sequences encoding the repeated motifs, RA1 and RA4, respectively located in the vicinity of the N- and C-terminal regions of the mature VspA protein and (iii) sequences encoding repeated motifs specific of *vspF*, RF, or specific of *vspE*, RE [8]. Fig. 1A–D illustrates the hybridization pattern obtained with genomic DNA extracted from Vsp-defined clonal variants (Fig. 1E) of type strain PG45 using the *vsp* oligonucleotide probes. In agreement with previously published data [8], the hybridization patterns indicate that (i) multiple DNA fragments hybridized with S, RA1 and RA4, respectively, (ii) DNA rearrangements occur at the *vsp* locus resulting in size polymorphism of *vsp*-associated DNA fragments among clonal variants derived from the same strain (Fig. 1 A–C) and (iii) sequences complementary to RE or RF occur as single copy on the chromosome with the variant shown in lane 4 lacking the RE sequence. Using a similar procedure, we assessed the distribution of related *vsp* sequences in 24 *M. bovis* field isolates. These represent four isolates randomly selected from isolates of group 1 and all 20 isolates representing group 2, 3 and 4, respectively. Since the *vsp* locus is subjected to spontaneous DNA rearrangements, field isolates that are the result of an unknown number of passages between animals, may be composed of cells that contain different *vsp* genomic configurations. In order to facilitate Southern blot analysis, field isolates were cloned three times prior to the DNA extraction, as recommended by Tully et al. [14]. Figs. 2–4 illustrate a set of identical

Table 1
Vsp-related antigens and *vsp*-related DNA sequences in *M. bovis* field isolates

Number of isolates	Expression of the 1E5 epitope[a]			Group	Presence of *vsp*-related DNA sequences[b]			
	Dot blot	Western blot	Colony blot		S	RA1	RA4	RE and RF
230	+	nd	nd	1	+	+	+	+ or −
10	−	+	nd	2	+	+	+	+
6	−	−	+	3	+	+	+	+
4	−	−	−	4	+	+	−	−

[a]Expression of the 1E5 epitope was assessed using the mAb 1E5 as described in Section 2.
[b]Presence of *vsp*-related DNA sequences was assessed by hybridization of *vsp*-specific oligonucleotide probes S, RA1, RA4, RE and RF with chromosomal DNA digested by *Hin*dIII, as described in Section 2. nd: not determined.

Southern blots in which *Hin*dIII-digested chromosomal DNA extracted from four cloned isolates of group 1 (Fig. 2), group 3 (Fig. 3) or group 4 (Fig. 4) was hybridized with individual *vsp* oligonucleotide probes. Hybridization profiles obtained with isolates of group 2 are not presented here, as they were very similar to those observed with group 1 or group 3. The results showed that for any given isolate, the hybridization pattern obtained with oligonucleotide S (Figs. 2–4A) is similar to that obtained with RA1 (Fig. 2C and Figs. 3 and 4B), suggesting a genetic link of these two sequences as also observed with defined clonal variants in Fig. 1 (A and B). Overall, the pattern generated with these two probes is more complex than that obtained with the clonal variants derived from *M. bovis* type strain PG45 (Fig. 1A,B), as it revealed a higher number of fragments with weaker and stronger intensities reflecting the number of repeated elements RA1 in a given DNA fragment. Detection of multiple DNA fragments that vary in their number and size from one field isolate to another indicates the presence of sequences homologous to the *vsp* genes in all *M. bovis* field isolates. Interestingly, comparison of profiles obtained with the oligonucleotide RA4 between isolates of groups 1 (Fig. 2B), 2 (not shown), 3 (Fig. 3C) and isolates of group 4 (Fig. 4C) revealed that isolates in which no immunostaining was obtained with mAb 1E5 failed

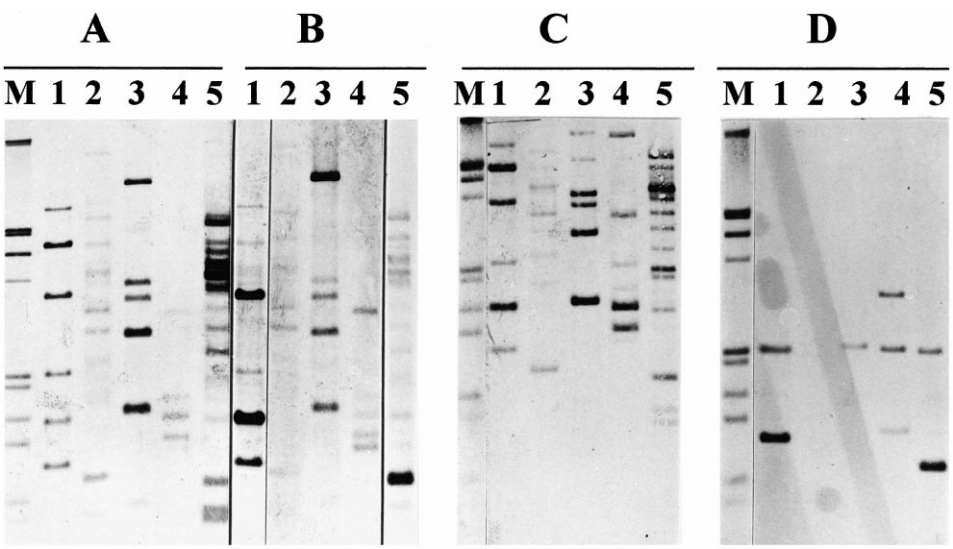

Fig. 3. Presence of *vsp*-associated *Hin*dIII DNA fragments in *M. bovis* field isolates of group 3. Chromosomal DNA extracted from a clonal isolate (lane 1, also depicted in Fig. 1, lane 1) and four *M. bovis* field isolates (lanes 2–5) randomly selected from group 3 (Table 1), was digested to completion with *Hin*dIII and hybridized with oligonucleotide probes S (A), RA1 (B), RA4 (C) or a combination of RE and RF (D). M, Molecular size marker in kpb depicted in Fig. 1.

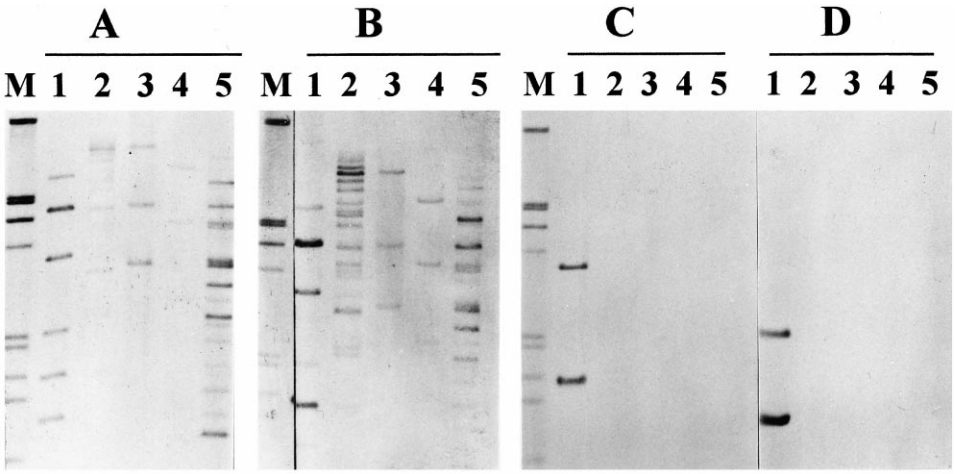

Fig. 4. Presence of *vsp*-associated *Hin*dIII DNA fragments in *M. bovis* field isolates of group 4. Chromosomal DNA extracted from a clonal isolate (lane 1, also depicted in Fig. 1, lane 1) and all four *M. bovis* field isolates (lanes 2–5) of group 4 (Table 1), was digested to completion with *Hin*dIII and hybridized with oligonucleotide probes S (A), RA1 (B), RA4 (C) or a combination of RE and RF (D). M, Molecular size marker in kpb depicted in Fig. 1.

to hybridize with the probe RA4, suggesting that the sequence RA4 may encode the 1E5 epitope. In these isolates, the DNA sequence that reacts with the RF probe is also lacking. However, there is no correlation between the *vspF* sequence and the sequence encoding the 1E5 epitope, as the VspE and the VspF products expressed in *Escherichia coli* both failed to react with the mAb 1E5 [8]. This is supported by results obtained with the two isolates shown in Fig. 2 (D, lane 3) and Fig. 3 (D, lane 2) that expressed the 1E5 epitope but did not hybridize with the RF and the RE probes.

4. Discussion

The results presented in this study revealed that *vsp*-related sequences occur in all *M. bovis* field isolates examined so far and that the number of *vsp* genes varies among isolates. Previous data have shown that in the type strain PG45 of *M. bovis* the *vsp* gene family is composed of eight *vsp* genes, including *vspA*, *vspB*, and *vspC* [8,9]. The complexity of the hybridization patterns obtained with the different *vsp*-specific probes indicates that in field isolates the number of *vsp* genes might not be limited to eight. In *M. bovis*, the *vsp* locus is subjected to high frequency DNA rearrangements that switch on and off expression of individual Vsp products resulting in organisms presenting a different antigen make up. This phenomenon that spontaneously occurs in a propagating population may account for the discrepancy of the results obtained when the field isolates were screened with mAb 1E5 using three different immunobinding assays. Failure to detect the 1E5 epitope by dot blotting in isolates of group 2 or by WB in isolates of group 3 may indicate that in these isolates the target epitope is only expressed by a small fraction of the population. If the degree of expression of the 1E5 epitope is associated with the frequency of DNA rearrangements that switch the expression of Vsps carrying this epitope, it is also dependent on the number of genes encoding the epitope. This might explain that in group 1 and 2, where the number of fragments hybridizing with the RA4 oligonucleotide is higher than in isolates of group 3, the epitope was expressed with a higher frequency. Finally, it appears that in isolates of group 4 the absence of the RA4 sequence is correlated with the complete lack of expression of the 1E5 epitope. This is in agreement with recent data showing that a truncated version of *vspA* lacking the RA4 repeated sequence is no longer reacting with the mAb 1E5 when expressed in *E. coli* (Brank, M., Citti, C. and Rosengarten, R., unpublished results). One puzzling result is the perfect genetic link ob-

served between the sequence corresponding to the signal peptide region and the sequence encoding the RA1 repeated motif of VspA that implies the presence of the RA1 sequence in each individual *vsp* gene. However, as shown for other mycoplasmas, families of variable surface proteins are often composed of several distinct members that share common blocks of amino acid sequence [18,19].

In a recent study, it was shown that sera collected from animals experimentally or naturally infected with *M. bovis* strongly reacted with epitopes carried by the Vsps, particularly with those of VspA and VspC, regardless of the clinical manifestations and the geographic location of the natural outbreaks (Le Grand, D., Citti, C., Bezille, P., Rosengarten, R. and Poumarat, F., unpublished results). These data taken together with those obtained in the present study that shows the presence of *vspA* or allelic versions of *vspA* genes in all field isolates tested so far, provide a solid basis for the development of serodiagnostic tools based on Vsp antigens.

5. Acknowledgements

The authors would like to thank F. De Simone (IZS, Brescia, Italy), J. Nicolet (University of Bern, Switzerland) and H. Pfützner (Jena, Germany) for providing isolates. This study is part of the European COST action 826 'Ruminants' mycoplasmoses' and was supported in part by a Grant (LPB/UBA/98/1/1) from the CNEVA and by the Austrian Ministry of Health and Consumer Protection (353.024/2-III/9/96).

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