Relationship between *Mycoplasma mycoides* subsp. *mycoides* ('Large-colony' Strains) and *M. mycoides* subsp. *capri*, as Indicated by Numerical Analysis of One-dimensional SDS-PAGE Protein Patterns

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Twenty-five strains classified as *Mycoplasma mycoides* subsp. *mycoides* LC or subsp. *capri* have been compared by one-dimensional SDS-PAGE of their cellular proteins. A computerized numerical analysis revealed that the protein patterns of all but two aberrant strains formed one large phenon that separated clearly from representatives of the four other members of the '*M. mycoides* cluster' at a similarity level (*S*) of 66% and which remained undivided at up to 78% *S*. At higher similarity levels, these strains fell heterogeneously into mixed sub-phenons containing strains of both subspecies. Serological comparisons by immunofluorescence largely confirmed the subspecies designations of the test strains, but also showed that some were serologically intermediate between subsp. *mycoides* and subsp. *capri*, being cross-reactive with both. These results confirm and enlarge upon those of our earlier studies indicating the protein-pattern inseparability of subsp. *capri* and subsp. *mycoides* LC strains and their distinctiveness from the classical *M. mycoides* subsp. *mycoides* SC strains and other members of the '*M. mycoides* cluster'. As also recognized by other workers, subsp. *mycoides* LC and subsp. *capri* strains appear to comprise one large group, wherein those most readily identifiable as either type lie at either end of a serological spectrum that also contains serologically cross-reactive strains. Our observations therefore suggest the lines along which the three groups classified at present within the species *M. mycoides* (SC and LC strains of subsp. *mycoides*; subsp. *capri*) might eventually be reclassified, subject to direct genomic comparisons.

INTRODUCTION

The species *Mycoplasma mycoides* at present contains two serologically-based subspecies, subsp. *mycoides* and subsp. *capri*. Strains distinguishable by serology as subsp. *mycoides* comprise two biological types (Cottew & Yeats, 1978). The so-called 'small-colony' (SC) strains of subsp. *mycoides* are found mainly in cattle, as the causative agent of the classical disease, contagious bovine pleuropneumonia. The other type, the 'large-colony' (LC) strains, are pathogenic for goats, their main host; they grow much more profusely and produce considerably larger colonies than the SC strains, despite their serological similarity. *M. mycoides* subsp. *capri*, also from goats, is serologically different from both SC and LC strains of subsp. *mycoides*, although very similar in cultural and biochemical characteristics to the LC strains. A complicating factor is that some isolates presumed to be the LC type do show serological cross-reactivity with *M. mycoides* subsp. *capri*, or vice versa, making their precise typing difficult (Cottew, 1979; Salih *et al.*, 1983; Nascimento *et al.*, 1986).

Abbreviation: IF, immunofluorescence.

The taxonomic relationships between the above three groups of mycoplasmas are therefore unclear. Furthermore, they fall within a larger 'problem-cluster' that also contains three other bovine or caprine/ovine mycoplasma groups – the so-called '*M. mycoides* cluster' (Cottew *et al.*, 1987). The overall relationships of all six groups have been examined previously by an analysis of their one-dimensional SDS-PAGE protein patterns (Costas *et al.*, 1987*a*). In that study, each of the six '*M. mycoides* cluster' groups was represented by the type/reference strain and a few other isolates. Despite their known serological differences, *M. mycoides* subsp. *capri* and the LC strains of subsp. *mycoides*, on the basis of their compared protein patterns, formed a single phenon, at the 70% similarity (*S*) level, that was distinct from that of the SC strains of subsp. *mycoides* and from all other 'cluster' members. Moreover, the subsp. *capri* and the LC strains could not be clearly separated from each other even at 75% *S*, where they fell heterogeneously into several sub-taxa. However, in that investigation, these two groups were each represented by only four or five strains and it was desirable to determine whether their apparent inseparability would be confirmed by similar studies with larger numbers of isolates of each group.

For the present study, further isolates were gathered that had already been classified elsewhere as either subsp. *capri* or subsp. *mycoides* LC, plus some strains provisionally classified as subsp. *capri* but serologically cross-reactive also with subsp. *mycoides* LC (Nascimento *et al.*, 1986). These additional strains, together with those previously tested, were checked serologically in this laboratory and all 25 strains were also examined together by one-dimensional SDS-PAGE. As previously, their protein patterns were compared in a computerized numerical analysis, in order to provide further insight into their possible taxonomic relationships.

METHODS

Mycoplasma strains. The 25 strains to be compared and their origins are shown in Table 1. These comprised eight strains from goats classified as *M. mycoides* subsp. *capri*, including the type strain PG3 (two samples), together with 12 goat strains classified as *M. mycoides* subsp. *mycoides* LC, including the reference strain, Y-goat, plus five Brazilian isolates (nos 22–26), tentatively classified as subsp. *capri* but serologically cross-reactive also with subsp. *mycoides* LC (Nascimento *et al.*, 1986). The type/reference strains for the other four members of the '*M. mycoides* cluster' (viz. *M. mycoides* subsp. *mycoides* SC, Bovine serogroup 7, F38 type and *M. capricolum*) were also included (Table 1, nos 27–30) for comparative purposes, providing reference 'markers' to our previous results (Costas *et al.*, 1987*a*). The sources of some of these strains have been indicated previously (Costas *et al.*, 1987*a*). Most of the others were provided through the courtesy of G. S. Cottew (LC and *capri* strains) and E. A. Freundt (Brazilian isolates of Nascimento *et al.*, 1986). Additional individual strains were kindly provided by A. J. Da Massa (GM12) and G. R. Smith (Pendik).

Serological tests. The mycoplasmas were compared serologically by a modification of the indirect immunofluorescence (IF) test applied to unfixed colonies on agar blocks (Rosendal & Black, 1972; Gardella *et al.*, 1983), using specific hyperimmune rabbit serum as the primary reagent and sheep anti-rabbit fluorescein isothiocyanate conjugate as the secondary reagent. The rabbit antisera used were specific reference sera prepared against *M. mycoides* subsp. *mycoides* SC ('Gladysdale'), subsp. *mycoides* LC (GM12) and subsp. *capri* (PG3). The 'Gladysdale' and PG3 antisera were kindly donated by R. M. Lemcke and the GM12 antiserum by A. J. Da Massa; the PG1 and alternative PG3 antisera referred to in Table 2 (footnote) were prepared in this laboratory.

Cultural and biochemical properties. All the test strains of *M. mycoides* subsp. *mycoides* LC and subsp. *capri* conformed in their general properties to those expected for these mycoplasmas, in that they all grew rapidly and profusely in broth, produced very large colonies on agar and produced strong acidity in glucose broth, but did not hydrolyse arginine (Cottew, 1979).

Preparation of protein samples. Glucose-broth cultures of each strain were grown and harvested to produce a cell pellet which was treated with double-strength lysis buffer. This was heated and centrifuged to give a supernatant containing the water-soluble proteins. Full details of the methods used to grow, harvest and prepare protein samples for electrophoresis have been published previously (Costas *et al.*, 1987*a*).

Electrophoresis. Discontinuous gels (16 × 18 × 0.15 cm) were cast to allow for 10 mm of stacking gel. The separation and stacking gels were prepared to give final polyacrylamide contents of 10% and 5% (w/v) respectively. Electrophoresis was done as described previously (Costas *et al.*, 1987*a*) for about 4 h in a Protean double-slab vertical electrophoresis cell (Bio-Rad) at a constant current of 30 mA per gel, until the marker dye had migrated 100 mm along the length of the separation gel.

Scanning of gels, analysis of data and computation of similarity. The stained protein patterns in the dried gels were scanned using an LKB 2222 Ultrosan XL laser densitometer (Pharmacia-LKB Biotechnology). Analysis and

Table 1. *Mycoplasma* strains used and their sources

Reference no. in dendrogram	Strain designation*	Country of isolation	Obtained from†
<i>M. mycoides</i> subsp. <i>capri</i>			
2; 20	PG3 (= NCTC 10137 ^T)	Turkey	2
1	N108	Nigeria	1
6	YC	Australia	1
8	ZZ	Australia	4
10	74.5907A	Australia	4
12	JM	Australia	1
14	Pendik	Turkey	4
19	BQT	Turkey	4
<i>M. mycoides</i> subsp. <i>mycoides</i> LC			
3	Y goat ^R	Australia	1
4	F30(M2055/75)	Kenya	5
5	VR1/3172.LB2	Australia	1
7	74/2488	Australia	4
9	81.636.1C	Australia	1
11	GE.6A.79E	Australia	1
13	KH1	Australia	1
15	Ojo 1	Nigeria	4
16	78/441	Australia	1
17	Cov 2	Turkey	4
18	GM12	USA	6
21	1217/77	Australia	1
<i>Brazilian isolates</i>			
22	G108/A2 clone b	Brazil	3
23	G108/A2 clone a	Brazil	3
24	G169/Leite	Brazil	3
25	G108/A3	Brazil	3
26	G105/A1	Brazil	3
<i>Other members of the 'M. mycoides cluster'</i>			
27	<i>M. mycoides</i> subsp. <i>mycoides</i> SC (PG1 = NCTC 10114 ^T)		2
28	Bovine serogroup 7 (N29 = NCTC 10133 ^R)		2
29	Strain F38 (NCTC 10192 ^R)		2
30	<i>M. capricolum</i> (California kid = NCTC 10154 ^T)		2

* T, type strain; R, reference strain for designated taxon.

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computation of similarity were based on the correlation coefficient and were essentially as published elsewhere (Costas *et al.*, 1989). Specific details of the parameter settings used in the analysis of PAGE protein patterns are available from the authors.

RESULTS

One-dimensional SDS-PAGE of whole-cell protein extracts of strains of *M. mycoides* subsp. *mycoides* LC and subsp. *capri* produced patterns containing over 50 discrete bands corresponding to molecular sizes of 18–100 kDa (Fig. 1). Protein samples of strain PG3 run on the two different gels used gave a similarity (*S*) value of 98%, indicating a high level of reproducibility for the study.

A numerical analysis comparing the protein patterns indicated that all but two of the strains previously assigned to *M. mycoides* subsp. *mycoides* LC and subsp. *capri* clustered together in one phenon that separated at 66% *S* and was undivided up to 78% *S*, as shown in the dendrogram (Fig. 2). The two strains that fell outside this large cluster were the subsp. *capri* strains, N108 and

Fig. 1. Electrophoretic protein patterns of *M. mycoides* subsp. *mycoides* LC and *capri* strains and related taxa of the '*M. mycoides* cluster'. The numbers placed horizontally refer to the strain references used in Table 1 and Fig. 2. The molecular mass markers (tracks labelled X) are (from top to bottom): ovotransferrin, 76-78 kDa; albumin, 66-25 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; myoglobin 17-2 kDa.

Fig. 2. Dendrogram of the cluster analysis based on total protein content of strains (vertical axis) listed in Table 1. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product moment correlation coefficient and UPGMA clustering. Numbers defined by the brackets are the sub-phenons formed at the 85% similarity level.

Pendik. Examination of their protein patterns (Fig. 1) indicates that the main differences accounting for their anomalous positions reside in the presence of an additional band at 36 kDa in the N108 pattern and an intense band at 24 kDa in the Pendik pattern, both of which were confirmed by repeat testing. Such heavy banding has been previously shown to cause strains to fall outside their appropriate cluster (Costas *et al.*, 1987*a*).

The 23 strains that were inseparable up to 78% *S* fell heterogeneously into further sub-phenons when compared at higher similarity levels. At 80% *S*, the majority of subsp. *capri* and subsp. *mycoides* LC strains fell into one of three sub-phenons and even at 85% *S*, where there were six sub-phenons, the largest was mixed, containing four subsp. *capri* and five subsp. *mycoides* LC strains (Fig. 2). It is interesting to note that all nine strains included in this sub-phenon were originally isolated in Australia, although isolates from that country were also found

Table 2. *Serological comparison of test strains*

Strain reference no. and designation	Titres obtained in indirect colony IF tests against antisera to:		
	* <i>M. mycoides</i> subsp. <i>mycoides</i> SC (Gladysdale†)	<i>M. mycoides</i> subsp. <i>mycoides</i> LC (GM12)	<i>M. mycoides</i> subsp. <i>capri</i> (PG3)
27 <i>M. mycoides</i> subsp. <i>mycoides</i> SC (PG1)	1000	400	< 10
<i>M. mycoides</i> subsp. <i>mycoides</i> LC			
3 Y goat	400	400	≤ 10
4 F30	1000	400	< 10
5 VR13712.LB2	200–400	200–400	10
7 74/2488	400	200	< 10
9 81.636.1C	40	20	10–40‡
11 GE.6A.79E	≥ 1000	400	80–100‡
13 KH1	1000	400	80
15 Ojo 1	400	400	< 10
16 78/441	200	100–200	< 10
17 COV 2	1000	400	< 10
18 GM12	400	1000	< 10
21 1217/77	400	200	10
<i>Brazilian isolates</i> (? subsp. <i>capri</i>)			
22 G108/A2 clone b	400	200	100–200
23 G108/A2 clone a	400	400–1000	400
24 G169/Leite	400	200	10
25 G108/A3	1000	400	200
26 G105/A1	400	200	100
<i>M. mycoides</i> subsp. <i>capri</i>			
2; 20 PG3	< 10	10	200
1 N108	≤ 10	200–400	100
6 YC	≤ 10	200	40–100
8 ZZ	< 10	≤ 10	100
10 74.5907A	< 10	< 10	1000
12 JM	< 10	< 10	400
14 Pendik	< 10	< 10	400–1000
19 BQT	< 10	≤ 10	200
30 <i>M. capricolum</i> (NCTC 10154)	< 10	< 10	< 10
28 <i>Bovine serogroup 7</i> (NCTC 10133)	20–40	50	< 10

* Results with a PG1 reference antiserum (homologous titre 100) not included, as it failed to react with other SC strains, including Gladysdale; it also gave substantially negative results (< 20) with all other test strains shown in this table.

† Homologous titre for Gladysdale: 400.

‡ These two strains gave negative results with an alternative PG3 antiserum prepared in this laboratory; for all the other test strains, this serum gave results similar to those shown above.

in other sub-phenons. Even at 90% *S*, there were two mixed clusters. There was no evident correlation between protein-pattern classification and the presumed identities of strains – either subsp. *mycoides* LC or subsp. *capri* – as determined, in traditional manner, on mainly serological grounds. Comparison of all 25 test strains by the colony-IF method (Table 2) in general confirmed the presumed serological identities. Thus, strains labelled as subsp. *capri* reacted significantly with subsp. *capri* antisera, but not with subsp. *mycoides* antiserum, and most of the subsp. *mycoides* LC strains reacted strongly with the subsp. *mycoides* LC antiserum and were largely unreactive with the subsp. *capri* antisera. There were some anomalies however in both groups (LC and *capri*) of test strains. Strain no. 11 (GE.6A.79E), known to be cross-reactive with subsp. *capri* (G. S. Cottew, personal communication), did show such cross-reactivity by IF,

as did strain no. 13 (KH1) unexpectedly, while strain no. 9 (81.636.1C), reportedly difficult to speciate (G. S. Cottew, personal communication), reacted only weakly with both subsp. *mycoides* and subsp. *capri* antisera. Among the nominal subsp. *capri* strains, there were two, nos 1 (N108) and 6 (YC), that were unexpectedly cross-reactive with subsp. *mycoides* LC antiserum while only moderately reactive with subsp. *capri* antisera. These various serological anomalies were not, however, reflected in any particular protein-pattern differences.

The Brazilian isolates (nos 22–26), provisionally classified as subsp. *capri* (Nascimento *et al.*, 1986), were confirmed to be cross-reactive also with antisera to subsp. *mycoides* LC and to the 'Gladysdale' antiserum but not to the type-strain antiserum for subsp. *mycoides* (PG1). One of these strains, no. 22 (M108/A2 clone b), which had some cultural and serological differences from the others (Nascimento *et al.*, 1986), was separated from them (but not from most subsp. *mycoides* LC and *capri* strains) in the protein pattern analysis at about 79% *S*. However, the other four separated as a discrete group at 86% *S* and were still inseparable at 90% *S* despite one of them, no. 24 (G169/Leite), being unlike the others in showing no reaction with subsp. *capri* antisera in our IF tests (Table 2).

All the test strains were markedly glucose-fermenting and grew profusely in all culture media, producing large colonies on solid medium, as expected of subsp. *capri* and *mycoides* LC strains (Cottew, 1979). The Brazilian isolates conformed to these characteristics, except for strain no. 22 (G108/A2 clone b), which produced more moderate growth and smaller colonies, despite being indistinguishable serologically from G108/A2 clone a (strain no. 23) and two other Brazilian isolates.

DISCUSSION

These results confirm and extend the findings previously reported (Costas *et al.*, 1987*a*) that most caprine mycoplasmas classified, on a serological basis, as either *M. mycoides* subsp. *mycoides* LC or subsp. *capri* could not be separated from each other on the basis of their one-dimensional SDS-PAGE protein patterns and mostly fell within one large phenon, embracing both subspecies. This phenon persisted at quite high levels of similarity and, not unexpectedly, also included strains that were serologically intermediate between the above two main groups. The phenon was also distinct from the representatives of the other four members of the '*M. mycoides* cluster', including notably PG1, the type strain of subsp. *mycoides* (SC). It should be noted that PG1 had a protein pattern that was characteristic of the subspecies, when previously compared with three other SC strains (Costas *et al.*, 1987*a*), despite its long passage history.

These findings provide further evidence in support of the concept that those *M. mycoides* strains that are more readily classifiable as either subsp. *mycoides* LC or subsp. *capri* fall at either end of a serological spectrum that also contains some serologically cross-reactive strains (Cottew, 1979), but whose members are all closely related in protein patterns. This concept supports the observations of Rodwell (1982; and as quoted by Cottew *et al.*, 1987) stressing the close protein-pattern congruence he noted in the two-dimensional PAGE patterns of a collection of strains representative of subsp. *mycoides* LC (14 isolates) and *capri* (seven isolates). Rodwell also emphasized the closer similarity between the subsp. *mycoides* LC and *capri* strains than between either of them and subsp. *mycoides* SC (seven strains). These two-dimensional PAGE observations were further supported by those of Nascimento *et al.* (1986) and conflict with other reports of the DNA and protein relationships of the three groups (Askaa *et al.*, 1978; Andersen *et al.*, 1984), which suggested that SC and LC strains of subsp. *mycoides* were more clearly related to each other than to subsp. *capri*. Those studies were, however, limited by their use of only a single representative strain for each of the groups. Furthermore, despite their serological differences, the subsp. *mycoides* LC and subsp. *capri* strains share several important biochemical and cultural characters that also distinguish them from the SC strains of subsp. *mycoides* (Cottew, 1979; Cottew *et al.*, 1987). We would expect, on the basis of the above and our own observations, that the reported DNA homology values between these three organisms would prove unrepresentative if a wider range of strains for each group were to be compared. Protein patterns obtained by one-dimensional SDS-PAGE provide an indirect measure of the coding

capacity of the mycoplasmal genome, and comparison of such patterns ought therefore to provide an approximate reflection of probable genomic relationships, as has been shown for other bacteria (Owen & Jackman, 1982; Costas *et al.*, 1987*b*; Roop *et al.*, 1985; Ursing & Firehammer, 1985). Comparative DNA-homology studies have been proposed (Cottew *et al.*, 1987) and are envisaged (Subcommittee, 1988) as part of an international programme intended to compare several representatives of each of the six members of the '*M. mycoides* cluster' by DNA homology.

In the meantime, our studies strongly point to the future inclusion of subsp. *capri*, together with the LC strains currently classified under subsp. *mycoides*, within one taxon, which our previous studies suggest would be separate from subsp. *mycoides* SC (Costas *et al.*, 1987*a*), and thus the classification of LC strains within subsp. *mycoides* would no longer be appropriate. Others have recognized the need for a revised classification of the three groups at present formally classified within the species *M. mycoides*, and various taxonomic schemes have been suggested, including the designation of LC and *capri* strains as serovars of subsp. *capri* (Salih *et al.*, 1983; Cottew *et al.*, 1987). This latter proposal would seem the most consistent with the results of our own one-dimensional SDS-PAGE protein pattern analyses.

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