Numerical Analysis of PAGE Protein Patterns and the Taxonomic Relationships within the '*Mycoplasma mycoides* Cluster'

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Twenty-six isolates belonging to the '*Mycoplasma mycoides* cluster' have been characterized by one-dimensional SDS-PAGE of their cellular proteins. A numerical classification based on the resulting patterns and using a correlation coefficient revealed four distinct phenons at a similarity (*S*) level of 70%, comprising: (a) bovine group 7 strains; (b) *M. capricolum* and F38-like strains; (c) *M. mycoides* subsp. *capri* and LC strains ('subsp. *mycoides*'); (d) *M. mycoides* subsp. *mycoides* (SC). At the 75% *S* level, they could be divided further to give eight phenons. The composition of the clusters at both levels was in good agreement with their previous classification, except for *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*, which were clustered in a single phenon at 70% *S* and could not be clearly separated at 75% *S*. We conclude that high-resolution SDS-PAGE, combined with computerized analysis of protein patterns, provides an extremely effective approach to the investigation of taxonomic relationships within this group of mycoplasmas.

INTRODUCTION

The sub-division of the genus *Mycoplasma* had originally seemed a relatively simple matter, most individual species being readily separable, mainly on a serological basis (Edward & Freundt, 1969; Freundt & Edward, 1979). In recent years, however, taxonomic problems have arisen, as the increasing number of known mycoplasmas has led to discovery of serological cross-reactivities between some presumed separate species. Genomic comparisons have also revealed other unsuspected inter-species relationships. Within *Mycoplasma*, the organisms known as the '*Mycoplasma mycoides* cluster' present the most prominent current taxonomic problem (Cottew *et al.*, 1987).

The '*Mycoplasma mycoides* cluster' contains mycoplasmas from cattle, sheep and goats, forming six different groups with varying degrees of genomic or antigenic inter-relationships. These groups include the named species *Mycoplasma mycoides* subsp. *mycoides* ['small-colony' (SC) strains, mainly from cattle], *M. mycoides* subsp. *capri* (goats) and *M. capricolum* (sheep and goats). The other three groups have uncertain taxonomic status. They include the so-called 'large-colony' (LC) strains (mainly from goats) tentatively classified as '*M. mycoides* subsp. *mycoides* (LC)', being serologically indistinguishable from the SC strains although biologically quite different (Cottew & Yeats, 1978). The two other groups in the cluster are as yet unnamed, viz. bovine serogroup 7 from cattle (Leach, 1973) and F38-related strains causing contagious pleuropneumonia in goats (Ernø *et al.*, 1983).

The complicated relationships, biological characters and aetiological associations of the six mycoplasma groups within this cluster are described and discussed in a recent report to the ICSB Subcommittee on the Taxonomy of Mollicutes (Cottew *et al.*, 1987). In brief, the main classification problems affecting the different members are as follows. (1) A close serological

Abbreviations: 1-D, one-dimensional; 2-D, two-dimensional; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

relationship between the *M. mycoides* subsp. *mycoides* SC strains and the LC strains, which are nevertheless quite distinct in their cultural, biochemical and pathogenicity properties. (2) Potential difficulties in distinguishing between the LC group and *M. mycoides* subsp. *capri*. (3) A close genomic relationship and partial serological cross-reactivity between the F38 group and *M. capricolum* and a very close serological similarity between F38 and bovine serogroup 7 strains, all three of these groups being quite distinct, however, in their general biochemical characters and in host relationships or pathogenicity.

The report to the Subcommittee (Cottew *et al.*, 1987) has stressed the potential taxonomic usefulness of two-dimensional (2-D) electrophoretic patterns (Rodwell, 1982) in pointing towards a suitable classification for these six groups, and also the need to rely ultimately on DNA homology tests in difficult cases and as a final means of resolving taxonomic discrepancies. However, both approaches entail considerable time and effort, especially as any comparison between individual groups ought to involve several isolates of each group, rather than merely the individual type or reference strains, as has been the tendency so far (Cottew *et al.*, 1987). The method that we have employed, a computerized numerical analysis of the one-dimensional (1-D) protein patterns of several strains for each of the six groups, has the advantage that all the test strains can be compared in one gel run, enabling a comprehensive comparative analysis of up to 30 strains under standard conditions and forming a programme readily achievable in weeks rather than the many months probably required for similar comparisons by the other methods.

METHODS

***Mycoplasma* strains.** Each of the six groups of the '*M. mycoides* cluster' was represented by three to five strains, including type or reference strains where appropriate, and *M. pneumoniae* was included as a control organism (Table 1). Cultures were grown at 37 °C in a liquid medium based on that of Hayflick (1965) and containing horse serum (20%, v/v), yeast extract (0.7%, w/v, Oxoid), thallos acetate (2.5%, w/v), glucose (1%, w/v), penicillin (200 U ml⁻¹) and phenol red (0.002%, w/v) in mycoplasma broth base (Oxoid) adjusted to pH 7.6.

Culture conditions. Broth cultures of each test strain were prepared by dilution (1/10, v/v) of an inoculum culture and reincubation until an appreciable acid colour change was observed.

Harvesting conditions. Appropriate amounts (25–100 ml) of test cultures were centrifuged for 30 min at 1300 g. Cell deposits were washed twice with phosphate-buffered saline (PBS; Dulbecco 'A', Oxoid; pH 7.3) and centrifuged again (30 min, 1300 g).

Preparation of protein samples. The resulting pellet was finally resuspended in about 60 µl double-strength lysis buffer [20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 4% (w/v) SDS and 70% (v/v) stacking gel buffer, see details below]. The thoroughly mixed bacterial sample in lysis buffer was incubated in a Pierce Reacti-therm heating module (Pierce Chemical Company) at 100 °C for 5 min. An equal quantity of sterile distilled water was added, and the tube was agitated and then heated at 100 °C for a further 5 min. The sample was then centrifuged (11 600 g) for 10 min to remove particulate matter and cellular debris. The supernatant containing water-soluble proteins was then removed carefully and stored at -20 °C.

Electrophoresis. Discontinuous gels (16 × 18 × 0.15 cm) were cast to allow for 10 mm of stacking gel. The separation gel was prepared to give a final polyacrylamide content of 10% (w/v) from a stock solution containing 29.2% (w/v) acrylamide and 0.8% (w/v) *N,N*-methylenebisacrylamide (30% T, 2.7% C_{bis}). The final concentrations of other components in the gel were 0.375 M-Tris/HCl (pH 8.8: prepared by adding 87.5 ml 5 M-HCl and made up to 1 litre with the Tris buffer solution) and 0.1% (w/v) SDS. Polymerization was achieved by adding 0.05% (w/v) ammonium persulphate (10%, w/v solution) and 0.05% (v/v) TEMED. The separation gel components excluding SDS were de-aerated for 3 min before polymerization, which took approximately 15 min at 20 °C. The stacking gel was prepared from the same stock solution used for the separation gel to give a polyacrylamide content of 5%. The remaining stacking gel components, including the stacking gel buffer, were added to give a final concentration of 0.125 M-Tris/HCl (pH 6.8: prepared by adding 95 ml 5 M-HCl and made up to 1 litre with the Tris buffer solution) and 0.1% (w/v) SDS. Polymerization was initiated by adding 0.05% (w/v) ammonium persulphate (10%, w/v solution) and 0.1% (v/v) TEMED. The stacking gel solution excluding SDS was de-aerated for 3 min and polymerization was evident after 6–8 min. The well-forming comb was removed from the stacking gel after 1 h and the sample wells were washed twice with tank buffer [0.25 M-Tris pH 8.3, 0.192 M-glycine, 0.1% (w/v) SDS]. The wells were then loaded with approximately 10 µl of the protein sample to which 0.001% (w/v, final concn) of bromophenol blue had been added as a marker dye.

Electrophoresis was carried out 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.

Table 1. *Mycoplasma* strains used and sources

Ref. no. in dendrogram	Strain designation*	Obtained from†	Country of isolation
<i>Bovine serogroup 7</i>			
1	L2917	1	Australia
2	QR1	1	Australia
3	NCTC 10133 ^R = N29 = PG50	2	Australia
<i>F38-like</i>			
4	G133 = C1668	3	Kenya
5	G183	4	Kenya
6	G275	4	Kenya
7	NCTC 10192 ^R = F38	5	Kenya
<i>M. capricolum</i>			
8	PP goat	6	Turkey
9	YP	1	Australia
10	YO	1	Australia
11	ZT	1	Australia
12	74/3220	1	Australia
13	NCTC 10154 ^T = California Kid	2	USA
<i>M. pneumoniae</i>			
14	NCTC 10119 ^T = FH	2	USA
<i>M. mycoides</i> subsp. <i>capri</i>			
15	74/5907A	4	Australia
16	ZZ	4	Australia
17	BQT	4	Turkey
18	NCTC 10137 ^T = PG3	2	Turkey
<i>M. mycoides</i> subsp. <i>mycoides</i> (LC)			
19	Y goat ^R	1	Australia
20	M2055/75 = F30	5	Kenya
21	74/2488	4	Australia
22	Cov 2	4	Turkey
23	Ojo 1	4	Nigeria
<i>M. mycoides</i> subsp. <i>mycoides</i> (SC)			
24	Gladysdale	4	Australia
25	Oremit	4	Kenya
26	KH3J	4	Sudan
27	NCTC 10114 ^T = PG1	2	

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

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A constant temperature of 10 °C was maintained throughout with a refrigerated recirculator (Coolflow CFT-33, Neslab Instruments). After electrophoresis, the gels were stained for 16 h in a staining solution containing 0.1% (w/v) Page Blue 83 (BDH) in an aqueous solution of methanol (25%, v/v) and acetic acid (10%, v/v). Gels were destained using the same solution, but with omission of the stain, until the background was clear, and were finally dried between dialysis membrane sheets (LKB 2003 slab gel dryer).

All chemicals used were the highest quality available (BDH). Acrylamide was Grade 1 quality; *N,N'*-methylenebisacrylamide, TEMED, ammonium persulphate, and Page Blue 83 were 'Electran' grade; SDS was 'especially pure' grade; Tris and glycine were 'Analar' grade; and the HCl was volumetric grade. The water used was deionized (resistance 10 MΩ cm⁻¹). BDH molecular mass markers (12.3 to 78 kDa) were included in all runs for calibration purposes.

Scanning of gels. The stained protein patterns in the dried gels were scanned (Ultrosan 2202 laser densitometer; LKB-Produkter) at a scan speed of 20 cm min⁻¹ and an absorbance range 0 to 1. The absorbance values were

recorded on magnetic disk (Apple Duodisk) as integers between 0 and 1000 using an interface program SCAN (Heyden Datasystems), and an Apple IIe microcomputer. The number of data points recorded along the length of the gel axis varied from 745 to 760 depending on the density of the protein bands on the original trace. The retention times and total areas of each protein band were calculated using an LKB 2220 recording integrator.

Computation. The raw data were carefully aligned and the initial (stacking gel/separation gel interface) and final (bromophenol blue marker) bands were deleted using Vidichart (Heyden Datasystems). The number of data points in each trace was reduced to a standard 500-point trace using an interpolation program (T PROCESS). A general background trend in each trace was removed to increase discrimination between patterns. The background cut-off was set at 0.6. Similarity between all possible pairs of traces was expressed using the Pearson product moment correlation coefficient. Strains were then clustered by the method of unweighted pair group average linkage (UPGMA) (Jackman *et al.*, 1983). Interpolation, background trend removal, calculation of similarity and clustering were all done on an Apple IIe microcomputer using programs (T PROCESS, T MATRIX, T CLUSTER) originally written in PET Basic but adapted to run in Applesoft Basic. Full details of the programs and mathematical treatment of background in T PROCESS are given elsewhere (Jackman *et al.*, 1983).

RESULTS

One-dimensional SDS-PAGE of whole-cell protein extracts of 26 strains belonging to the '*Mycoplasma mycoides* cluster' (as defined in this study) and the reference strain of *M. pneumoniae* produced patterns containing 50–55 discrete bands corresponding to molecular sizes of 18 to 100 kDa (Fig. 1*a, b*). Duplicate samples of the protein molecular mass marker set, run on different gels, gave a correlation coefficient (r) of $95 \pm 1\%$, indicating a high reproducibility for the method. In addition, duplicate samples of the mycoplasma proteins run on different gels gave r values of 90–94%, again indicating high reproducibility.

A numerical analysis (using the Pearson product moment correlation coefficient and UPGMA clustering) revealed four distinct clusters (phenons) at a similarity (S) level of 70%, only two strains (*M. capricolum* YP and *M. pneumoniae* NCTC 10119¹) remaining outside these clusters, as indicated in the dendrogram (Fig. 2). Although the protein patterns of all 26 '*M. mycoides* cluster' strains were generally similar, with many shared common bands, each of the four phenons (1–4) also had proteins common only to its members and not shared by the other phenons. A degree of heterogeneity was also evident within each phenon and especially within phenons 2 and 3.

Comparison at the 75% S level gave a total of eight phenons, resulting entirely from division of phenons 2 (into 2a, 2b and 2c) and 3 (into 3a, 3b and 3c). The average inter- and intra-phenon similarities are listed in Table 2. The phenons recognized proved to be extremely stable when computations were repeated using different levels of trace alignment and background subtraction. The features of the various phenons are described below.

Phenon 1 (bovine group 7). All three strains of bovine group 7 mycoplasmas fell into phenon 1. The intra-group average similarity was $87.0 \pm 3.7\%$, indicating a relatively homogeneous group with respect to overall protein pattern. The strains produced a distinctive high-intensity region (46–50 kDa) (Fig. 1*a, b*), comprising four overlapping bands (46.1, 46.8, 48.2 and 49.4 kDa) that accounted for 20–25% of the total protein content of each strain. Several of these bands were also represented in the protein profiles of other phenons (see below). A secondary band pattern, differing significantly from that of other phenons within the '*M. mycoides* cluster', included bands at 65.2, 60.0 and 54.4–53.5 kDa in the high molecular mass region and 43.3, 37.5, 35.2, 26.4 and 21.4 kDa in the lower molecular mass region. Most of these positions were also represented in the patterns of strains in the other three phenons, but the 26.4 kDa band appeared to be unique to the three bovine group 7 strains. The nearest neighbours of phenon 1 in terms of average similarity were phenon 2b (*M. capricolum*) and phenon 3a (*M. mycoides* subsp. *mycoides* LC/*M. mycoides* subsp. *capri*) with which it had similarities of 72.6 and 70.6%, respectively.

Phenon 2 (F38/*M. capricolum*). At the 70% S level, nine of the ten strains previously assigned to these groups fell into phenon 2, the single exception being strain YP of *M. capricolum*, which remained unclustered. The high intra-phenon standard deviation (SD) of 8.2% and low average similarity of its members (77.5%) reflected the appreciable degree of heterogeneity in protein patterns of this phenon.

Fig. 1(a, b). Electrophoretic protein patterns of '*Mycoplasma mycoides* cluster' strains. The numbers placed horizontally are the strain reference numbers used in Table 1 and Fig. 2. Molecular sizes are indicated vertically in kDa. The molecular mass marker set (tracks labelled X) are (from top to bottom): ovotransferrin (76-78 kDa), albumin (66-25 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25.7 kDa) and 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. The indicated phenons are associated with the following: 1, bovine serogroup 7; 2a, F38-like group; 2b and 2c, *M. capricolum*; 3a, 3b and 3c, *M. mycoides* subsp. *mycoides* (LC strains) and subsp. *capri*; 4, *M. mycoides* subsp. *mycoides* (SC strains).

At the 75% *S* level, phenon 2 could be further divided into three sub-phenons (a-c). Phenon 2a consisted of all four strains of the F38-like group, including the reference strain F38 (NCTC 10192) and three other strains previously identified on the basis of serological and biochemical properties. The intra-phenon average *S* level for phenon 2a was $88.3 \pm 2.8\%$, confirming the homogeneity of the group. The protein patterns were in general similar to those of phenons 2b and 2c (comprising *M. capricolum* strains), with which the F38-like (phenon 2a) group strains had average similarities of 74.8 and 72.8% respectively. The major difference between the three phenons (2a, 2b and 2c) was the lower overall intensity of banding in the 46–50 kDa region of the F38 group (phenon 2a) strains, characterized by bands at 46.1, 48.2 and 49.4 kDa. These bands accounted for 15% of the total proteins, with the band at 46.1 kDa contributing only 1–2% of the total proteins, in contrast to the figure of > 5% for bovine group 7 mycoplasma (phenon 1), *M. capricolum* (phenons 2b and c) and *M. mycoides* subsp. *mycoides* LC/*M. mycoides* subsp. *capri* (phenon 3).

Table 2. Mean intra- and inter-phenon percentage similarities ($\pm$ SD) as determined by the Pearson product moment correlation coefficient (r) and UPGMA clustering (all phenons are formed at the 75% S level)

	Phenon 1 ($n=3$)	Phenon 2a ($n=4$)	Phenon 2b ($n=4$)	Phenon 2c ($n=1$)	Phenon 3a ($n=3$)	Phenon 3b ($n=4$)	Phenon 3c ($n=2$)	Phenon 4 ($n=4$)	Strain 9 ($n=1$)	Strain 14 ($n=1$)
Phenon 1	87.0 $\pm$ 3.7									
Phenon 2a	68.6 $\pm$ 3.3	88.3 $\pm$ 2.8								
Phenon 2b	72.6 $\pm$ 6.0	74.8 $\pm$ 7.0	82.2 $\pm$ 5.3							
Phenon 2c	63.3 $\pm$ 2.4	72.8 $\pm$ 4.3	69.5 $\pm$ 4.7	(100)*						
Phenon 3a	70.6 $\pm$ 3.6	63.3 $\pm$ 4.0	69.8 $\pm$ 3.7	60.3 $\pm$ 3.7	81.7 $\pm$ 0.9					
Phenon 3b	67.1 $\pm$ 5.0	69.4 $\pm$ 4.4	67.4 $\pm$ 4.6	61.3 $\pm$ 1.5	75.3 $\pm$ 3.0	83.8 $\pm$ 3.5				
Phenon 3c	55.5 $\pm$ 4.5	59.9 $\pm$ 3.7	62.9 $\pm$ 2.4	62.0 $\pm$ 0.0	71.7 $\pm$ 5.4	71.0 $\pm$ 3.1	78.0			
Phenon 4	62.6 $\pm$ 8.5	66.6 $\pm$ 5.7	59.0 $\pm$ 7.1	55.0 $\pm$ 10.8	60.2 $\pm$ 6.6	66.9 $\pm$ 4.6	50.5 $\pm$ 6.2	78.8 $\pm$ 4.4		
Strain 9	59.0 $\pm$ 4.6	61.5 $\pm$ 2.9	69.8 $\pm$ 3.3	60	55.0 $\pm$ 2.2	50.5 $\pm$ 2.2	47.0 $\pm$ 3.0	49.3 $\pm$ 3.3	(100)*	
Strain 14	14.7 $\pm$ 2.8	12.3 $\pm$ 2.9	11.3 $\pm$ 5.0	10	15.0 $\pm$ 1.6	9.3 $\pm$ 4.8	14.0 $\pm$ 3.0	7.5 $\pm$ 3.5	10	(100)*

 n , Number of strains in phenon.

* Only one strain included in phenon.

Phenon 2b comprised four of the six *M. capricolum* strains and phenon 2c contained only one strain, the type strain (NCTC 10154) for the species *M. capricolum*. Both phenons were similar to each other and to phenon 2a, sharing many common bands as reflected in the high inter-phenon similarities (Table 2). However, phenon 2c did differ significantly from both 2a and 2b in its secondary banding pattern, having a more intense band at 37 kDa (~12% of total protein) as compared with a band of similar position, 37.4 kDa, in phenons 2a and 2b (~5%). In contrast, the band at 35.2 kDa was weak for phenon 2c (<2%), but more intense for phenons 2b (6.10%) and 2a (2.6%).

Phenon 3 (*M. mycoides* subsp. *mycoides* LC and subsp. *capri*). The nine strains included in phenon 3 comprised all of those previously classified as either *M. mycoides* subsp. *mycoides* LC or *M. mycoides* subsp. *capri*. The intra-phenon average similarity of 75.8–5.8% indicated some heterogeneity within phenon 3. At the 75% *S* level, this phenon could be further divided into three phenons (Fig. 2). The distribution of the nine strains among these three phenons appeared unrelated to previous identity: strains of *M. mycoides* subsp. *mycoides* LC were represented in 3a and 3b, and comprised 3c, whereas strains of *M. mycoides* subsp. *capri* were found only in 3a and 3b. The intra-phenon average similarities for phenons 3a, 3b and 3c were 81.7, 83.8 and 78% respectively, each thus showing an increase in homogeneity over that of the undivided phenon. The inter-phenon similarities, however, remained high (all >70%).

There appeared to be a greater diversity of patterns within phenon 3 than in phenon 2, where diversity was due more to quantitative than to qualitative differences. This is seen clearly in the 47–37 kDa region of the phenon 3 patterns (Fig. 1*b*) where most of the diversity is found. The secondary banding pattern of phenon 3 also differed notably from phenons 1 and 2 in the lack of a band at 32.8 kDa.

Phenon 4 (*M. mycoides* subsp. *mycoides* SC). Phenon 4 contained only strains previously identified as *M. mycoides* subsp. *mycoides* SC, including the type strain PG1 (NCTC 10114). The average intra-phenon similarity was $78.8 \pm 4.4\%$. The protein patterns were in general similar to those of the other phenons represented in this study, but with major differences in the banding region, 46–50 kDa, within which there was only one relatively intense band (49.4 kDa), which accounted for 6–9% of the total protein content. Although bands at 48.2 and 46.1 kDa were also present within this region, they were weak (<2–3%) as compared with corresponding bands for other phenons.

M. capricolum (strain YP). This strain did not cluster with any of the phenons at either 70 or 75% *S*. Its pattern was, however, generally similar to those of *M. capricolum* phenon 2b strains, except for a distinctive intense band (66.5 kDa) accounting for 25% of the total protein and present exclusively in the pattern of this strain. A second analysis, excluding the band at 66.5 kDa, that compared *M. capricolum* strain YP with all other strains gave average similarities of 69.6, 73.3, 83.5, 61.2 and 55.5% with phenons 1, 2a, 2b, 3 and 4 respectively. This analysis suggests that the presence of the heavy band at 66.5 kDa in *M. capricolum* YP had weighted the first analysis away from a clustering of this strain with members of the same species. The second analysis indicated that *M. capricolum* strain YP is most similar to phenon 2b (*M. capricolum*).

DISCUSSION

As at present envisaged (Cottew *et al.*, 1987), the '*M. mycoides* cluster' comprises six distinguishable mycoplasma groups, two being unnamed and four belonging to named species or subspecies. In our numerical analysis of 1-D SDS-PAGE protein patterns, all but one of the 26 strains of the cluster examined fell into taxonomic clusters that conformed with the existing classification at the 75% *S* level, except that strains hitherto classified separately as *M. mycoides* subsp. *mycoides* LC and subsp. *capri* could not be clearly distinguished from each other.

Phenons 1 and 2. The taxonomic position of bovine group 7 strains (phenon 1) has been problematical, largely because of the uncertain relationship of this group with *M. mycoides*

(Leach, 1973). Although at the 70% *S* level the three representatives of this group that we examined fell into a cluster also containing F38 and *M. capricolum*, they formed a separate phenon, distinct from all others, at the 75% *S* level. This result is compatible with their being classified as a distinct species, as suggested by Salih *et al.* (1983) on the basis of the characteristic isoenzyme patterns of bovine group 7. Such a classification is consistent with reported DNA/DNA hybridization values of 58–64% between group 7 and other members of the '*M. mycoides* cluster' (Christiansen & Ernø, 1982), although a single higher value of 85% homology with *M. mycoides* subsp. *mycoides* SC has been reported (Askaa *et al.*, 1978). Protein congruence determined from 2-D PAGE studies (Andersen *et al.*, 1984; Nascimento *et al.*, 1986; Rodwell, 1982) also places bovine group 7 strains roughly equidistant from the other cluster members. However, A. W. Rodwell's (personal communication) later revision of his data places bovine group 7 slightly closer to *M. capricolum*, in closer agreement with our present results (Fig. 2), which at the 70% *S* level place bovine group 7 (phenon 1) closest (69.7% *S*) to phenon 2 containing *M. capricolum* and F38 strains. The reported serological cross-reactivity of bovine group 7 with *M. capricolum* (Ernø *et al.*, 1983) and with F38 (Cottew *et al.*, 1987) endorses this limited relationship, but the overall evidence at present suggests bovine group 7 to be a separate taxon, although with similarities to *M. capricolum* and F38.

The DNA homology value for the *M. capricolum* and F38 type/reference strains was reported as 80% (Christiansen & Ernø, 1982), this high value being consistent with their high protein pattern congruence (Andersen *et al.*, 1984; Rodwell, 1982 and personal communication). Our PAGE findings are in general agreement with these reports in that the two mycoplasma groups constitute a single phenon (2) at the 70% *S* level but divide into separate phenons, 2a (F38), 2b and 2c (*M. capricolum*), at 75% *S*. The single anomalous unclustered strain (YP) of *M. capricolum* proved to be most similar (83.5% *S*) to the other five *M. capricolum* strains when a re-analysis that disregarded the 66.5 kDa protein band was performed. This emphasizes the possibility of misidentification at and below the species level, if total protein PAGE patterns alone are used, with coefficients employing both qualitative and quantitative data, as already noted for *Providencia* spp. (Costas *et al.*, 1987; Holmes *et al.*, 1987). Although certain biochemical and growth characteristics serve to separate the *M. capricolum* and F38 groups, there are serological similarities between some strains of each (Ernø *et al.*, 1983; Cottew *et al.*, 1987). These and their genomic similarities, taken together with our protein pattern results, tend to place both within a single taxon, but as distinguishable groups, in accordance with the suggestion (Christiansen & Ernø, 1982) of F38-like strains as a possible subspecies of *M. capricolum*. Further DNA homology studies on selected representatives of these two groups, as recently proposed (Cottew *et al.*, 1987), should help to resolve finally this taxonomic issue, which has important veterinary implications.

Phenons 3 and 4. With regard to mycoplasmas at present comprising the species *M. mycoides*, the striking result is that, even at low levels of similarity (<65% *S*), all four strains of the classical SC type of subsp. *mycoides* were clearly separable (as phenon 4) from all other groups and were quite distinct from phenon 3 containing both subsp. *capri* and the LC strains at present assigned to subsp. *mycoides*. These two groups formed a single phenon (3) at the 70% *S* level and could not be clearly separated from each other even at the 75% *S* level, where they fell heterogeneously into three sub-phenons. The type/reference strains of subsp. *mycoides*, SC (PG1) and LC (Y goat), and of subsp. *capri* (PG3) were reported (Askaa *et al.*, 1978) to be closely interrelated by DNA hybridization comparisons, with values of 90% for Y goat and PG1 and 80% for PG1 and PG3, although somewhat lower values (75%) have been found for the latter pair (Andersen *et al.*, 1984). Contrary to these results but consistent with ours, an extensive analysis of 28 strains by 2-D PAGE protein patterns (Rodwell, 1982) indicated that the LC strains of subsp. *mycoides* are more closely related to subsp. *capri* than to SC strains of subsp. *mycoides*. Nascimento *et al.* (1986) endorsed this finding in studies of field isolates from cases of caprine mycoplasmosis and also demonstrated the existence of strains intermediate between subsp. *mycoides* LC and *capri*, and less related to subsp. *mycoides* SC, both serologically and by their 2-D protein patterns. Serological difficulties in distinguishing between some LC and *capri*

strains are not surprising, in view of the complete overlap between the two groups in our results. It seems likely that, in the past, the allocation of isolates to one or other subspecies of *M. mycoides*, and particularly to subsp. *capri*, might sometimes have been somewhat arbitrary and perhaps influenced by the clinical background. The taxonomic confusion between these two groups is evident, in that two of the three sub-phenons they formed at the 75% *S* level contained strains from each group. More extensive 1-D PAGE studies, with a wider range of isolates representing these two groups, are underway, but it is already evident that some taxonomic revision will be needed that will reflect their close similarity. Such a reclassification should probably include the LC and subsp. *capri* strains within a single major taxon clearly separate from that of the SC (subsp. *mycoides*) strains. The taxonomic level of this separation would best be determined by more extensive DNA-homology studies on each group. Meanwhile, we are extending the comparisons between the two groups by further 1-D PAGE studies on a wider selection of isolates from each.

In conclusion, our analysis of 1-D protein patterns of the '*M. mycoides* cluster' throws some light on the main problem areas as outlined in the Introduction. (1) The classical SC strains of *M. mycoides* subsp. *mycoides* are distinct from the LC strains, which ought therefore to be separated from this subspecies. (2) The close relationship between the LC strains and *M. mycoides* subsp. *capri* is confirmed, but requires further analysis. (3) Bovine group 7 strains appear to have distinctive protein patterns, although being most similar to *M. capricolum* and F38 strains, which are themselves closely related. We interpret our results as pointing towards the future division of the '*M. mycoides* cluster' into four major taxa, conceivably separate species, and comprising: (a) SC strains of *M. mycoides* subsp. *mycoides*; (b) LC strains of subsp. *mycoides*, together with those of subsp. *capri* (further sub-division of this taxon to be determined); (c) *M. capricolum* together with F38-type strains, each as potential subspecies; (d) bovine group 7 strains. Authoritative decisions on final taxonomy will await further studies, including more comprehensive PAGE studies of protein patterns and, more particularly, DNA-homology studies upon a wider selection of strains from each of the existing six groups of the '*M. mycoides* cluster'. Meanwhile, at a more practical level, it is apparent that numerical analysis of 1-D SDS-PAGE patterns of mycoplasmal cellular proteins provides a useful approach towards clarifying relationships within the '*M. mycoides* cluster'.

We would emphasize that a high level of pattern reproducibility, both within and between gels, is essential for such studies to be reliable and that this can be attained if the methods described here are adhered to strictly.

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