Relationships Between Strains of *Mycoplasma mycoides* subsp. *mycoides* and *capri* Studied by Two-dimensional Gel Electrophoresis of Cell Proteins

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Strains of *Mycoplasma mycoides* subsp. *mycoides* have been divided into small colony (SC) and large colony (LC) types (Cottew & Yeats, 1978). The protein patterns of representative strains of these two types and of *M. mycoides* subsp. *capri* were compared by a high resolution, two-dimensional gel electrophoretic method. The results suggest that the LC strains are more closely related to *M. mycoides* subsp. *capri* than to the SC strains of *M. mycoides* subsp. *mycoides*.

INTRODUCTION

The separation of *Mycoplasma mycoides* into the subspecies *mycoides* and *capri* rests solely on the nature of their carbohydrate antigenic determinants: the former subspecies synthesizes a polysaccharide having 6-O- β -linked D-galactofuranoside determinants (Buttery, 1970) and the latter, a glucan (Jones *et al.*, 1965). Cottew & Yeats (1978) have separated the subsp. *mycoides* into two types on the basis of growth rate, colony size and other characteristics. Strains of the organism of bovine pleuropneumonia and a few strains isolated from goats are of the slower-growing small colony (SC) type. The faster-growing large colony (LC) type are mostly strains isolated from goats.

Organisms of the LC type resemble those of *M. mycoides* subsp. *capri* by the criteria of Cottew & Yeats (1978), and also in their propensity for growth in the ρ -form (Rodwell *et al.*, 1973). Under appropriate conditions ρ -forms were abundant in all recently isolated strains of the LC type and also of the subsp. *capri* but were not found in SC strains. Thus, apart from the difference in antigenic determinants, strains of the LC type resemble the *capri* subsp. rather than SC strains. Hence it was of interest to examine the phylogenetic relationships between the two subspecies by other means.

In this paper we compare the protein 'fingerprints' of representative strains by a high resolution, two-dimensional gel electrophoretic method. We conclude that strains of the LC type are more closely related to *M. mycoides* subsp. *capri* than to the SC type of *M. mycoides* subsp. *mycoides*.

METHODS

***Mycoplasma* strains.** The strains and their classification are shown in Table 1. Apart from strain H_{22/16}, the subsp. *mycoides* strains were those used by Cottew & Yeats (1978) in reaching their criteria for subdivision into SC and LC types. Of the SC strains, PG1 (type strain for the *mycoides* subsp.), V5, Gladysdale and KH₃J were from cattle and the O, P and Vom strains were from goats. Of the LC strains, H_{22/16} is a ρ -form mycoplasma with the characteristics of the LC type which was isolated after enrichment by serial subculture of the V5 strain in a partly defined medium (Rodwell *et al.*, 1972); strain 801 was isolated from the eye of a cow and the other strains were from goats. PG3 is the type strain for the *capri* subsp., while 5907A and 5357L were isolated in Australia from goats (Littlejohns & Cottew, 1977).

Growth of organisms. The growth medium (pH 7.8) contained: PPLO broth base without crystal violet (Difco), 2.1 % (w/v); Albimi yeast autolysate (Pfizer), 0.6 % (w/v); DNA (calf thymus), 0.002 % (w/v); sodium phosphate, 0.05 M; glucose, 0.04 M; ox serum, 10 % (v/v). The medium was sterilized by filtration through Seitz pads.

The organisms were grown in 5 ml portions of medium containing [^{14}C]protein hydrolysate ($2.5 \mu\text{Ci ml}^{-1}$) and harvested in the late-exponential or early-stationary phase of growth as determined by absorbance at 650 nm. The cells were washed once in 5 ml of a solution containing 0.15 M-sodium phosphate, 10 mM- MgSO_4 and 1 % (w/v) Casamino acids (Difco) at pH 7.0. The yield of protein varied from 0.25 to 0.5 mg for the SC strains and from 1.0 to 2.0 mg for the LC and subsp. *capri* strains.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was carried out by the method of O'Farrell (1975) in which proteins are separated in the first dimension by isoelectric focusing in gel cylinders and in the second dimension by sodium dodecyl sulphate-gel electrophoresis in gel slabs, with modifications to improve the solubilization and focusing of proteins in the first dimension. The modified electrophoretic method and the method used for sample preparation have been described (Archer *et al.*, 1978). Samples applied to the isoelectric focusing gels contained 25000 d.p.m. of radioactivity and about 20 μg protein. The volume of the samples varied from 10 to 25 μl . The resolving gel for SDS-gel electrophoresis in the second dimension contained a uniform 12 % (w/v) concentration of acrylamide. The protein spots were detected by the fluorographic method of Bonner & Laskey (1974) as modified by Laskey & Mills (1975).

Comparison of protein patterns. Comparison of the protein patterns between pairs of strains was facilitated by using positive copies of the fluorograms in which the protein spots appeared as translucent spots on a dark background. The positive copy of the fluorogram for one strain was placed over that of another and the common spots were marked on the top film. The positive copies were then interchanged, top to bottom, and the common spots were marked on the new top film. The numbers of marked spots and the total number of spots on each film were used to determine the percentage congruence between the two patterns (Table 1).

RESULTS AND DISCUSSION

The type strains for the subsp. *mycoides* and *capri* are PG1 and PG3, respectively. Strain Y was selected as a standard strain for the LC type of the *mycoides* subsp. Fluorograms of these three strains after exposure at -70°C for 10 d (Fig. 1*a*, *b* and *c*) contained 132, 142 and 154 spots, respectively. After an optimum exposure of about 20 d, approximately 300 individual spots were counted on fluorograms of strains of *M. mycoides*. This represents about half of the expected coding capacity of the genome. Autoradiographic spreading of spots during prolonged exposure is a major factor limiting resolution: minor spots lying close to major ones may be engulfed before they can be detected. Where we saw a series of spots on the same horizontal ordinate but of similar pI values we interpreted this as an example of charge heterogeneity of a single protein. An example can be seen in Fig. 1(*a*) at the coordinates (horizontal $\times$ vertical) 49×14 . Charge heterogeneity may be induced *in vivo* by post-transcriptional modification of proteins or *in vitro* during sample preparation and analysis (O'Farrell, 1975).

All cultures of the LC strains of the *mycoides* subsp. and of the *capri* subsp. contained a high proportion of ρ -forms (Peterson *et al.*, 1973). Protein B, the protein of which the ρ -fibre is composed (Rodwell *et al.*, 1975), is located at the coordinates 65×85 , 70×74 and 70×85 in Fig. 1(*b*, *c* and *d*), respectively. Protein A, located at the coordinates 60×27 and 65×25 in Fig. 1(*b* and *d*), respectively, is a major, membrane-associated protein (Rodwell *et al.*, 1975) which is present in most, but not all, strains of *M. mycoides* subsp. *mycoides* of the LC type and of subsp. *capri* (Rodwell *et al.*, 1973). It was not synthesized by any of the SC strains.

The percentage congruence of protein pattern between the various strains and each of the three standard strains was determined on fluorograms exposed for 4 d, because the task of matching spots was easier with the less complex patterns and the smaller spots. The total number of spots counted on the films ranged from 70 to 120. In some cases the percentage congruence was also determined using fluorograms exposed for 30 d. These results were similar to those obtained after the shorter exposure. Although the patterns showed excellent agreement between replicate analyses, the absolute positions of the spots varied slightly.

Fig. 1. Protein patterns of *Mycoplasma mycoides* strains: (a) PG1, (b) Y, (c) PG3, (d) 801. Fluorograms were exposed at -70°C for 10 d. Horizontal ordinate, first dimension by isoelectric focusing (IEF); vertical ordinate, second dimension by SDS-gel electrophoresis. Samples were applied to the IEF gels at the cathode (left). A small amount of protein (5 to 10 %) failed to enter the IEF gels and appears as a vertical streak in the second dimension. Spots are located by the intersection of the horizontal and vertical coordinates, the scales being in millimetres on the original fluorograms. Spots referred to in the text are indicated by arrows or crossed arrows.

Table 1. *Percentage congruence* of protein patterns between the standard strains and other strains of Mycoplasma mycoides subsp. mycoides and capri*

Strain	Standard strain		
	PG1	Y	PG3
subsp. <i>mycoides</i> SC type†			
PG1	—	< 10	< 10
V5	83	< 10	< 10
Gladysdale	78	< 10	< 10
KH ₃ J	82	< 10	< 10
O	67	< 10	< 10
P	58	< 10	< 10
Vom	79	< 10	< 10
subsp. <i>mycoides</i> LC type†			
Y	< 10	—	26
H _{22/1t}	< 10	100	27
801	< 10	99	26
OSB42	< 10	45	18
KH1	< 10	60	29
Ojo1	< 10	40	30
Ojo2	< 10	50	41
74/2488	< 10	63	30
Cov	< 10	31	39
subsp. <i>capri</i>			
PG3	< 10	26	—
5907A	< 10	33	47
5357L	< 10	37	40

* Percentage congruence was calculated as $[2N_{AB}/(N_A + N_B)] \times 100$, where N_A and N_B were the total numbers of spots on the fluorograms for strains A and B, and N_{AB} was the number of spots common to both.

† Small colony (SC) and large colony (LC) types (Cottew & Yeats, 1978).

Thus, when spots in different areas of the fluorograms were being matched it was necessary to move one film relative to the other over distances of a few millimetres. Matching of spots was governed by pattern recognition, i.e. by the position of the spot relative to its neighbours and by spot size and shape, and where any two strains had enough protein pattern in common, spots could be matched with confidence. When the SC strains were compared with strains Y or PG3, and when the LC strains or subsp. *capri* strains were compared with PG1, there was insufficient pattern in common to make spot-matching possible. The value (less than 10%) assigned for the congruence of pattern in these comparisons represents an upper limit and congruence may well be much lower.

The results (Table 1) showed:

(i) There was a significant congruence between the protein patterns of the strains within each of the three groups and their respective standard strains, whereas congruence could not be shown between the protein patterns of the SC strains and strains Y or PG3, or between the LC and subsp. *capri* strains and PG1.

(ii) A congruence of pattern ranging from 18 to 41% was found between the LC strains and PG3, and from 26 to 37% between the subsp. *capri* strains and strain Y. The strain Cov occupied an intermediate position, its protein pattern being a little more closely related to PG3 than to strain Y.

(iii) The protein patterns of strains Y and H_{22/1t} were identical, even after exposure for 30 d. Thus our earlier suggestion (Rodwell *et al.*, 1972) that the *p*-form strain H_{22/1t} was a variant derived from the V5 strain cannot be correct: strain H_{22/1t} could not possibly have been derived by short-term mutation and selection from the V5 strain and must have originated by cross-contamination of a culture of V5, probably with strain Y. As a consequence

of its faster growth rate in the partly-defined medium, the contaminant would have then been enriched by serial subculture and isolated after cloning.

(iv) Strain 801 is very closely related to, but not identical with strain Y (congruence of pattern 99%). It synthesized three proteins (indicated by crossed arrows in Fig. 1*d*) which were absent from the fluorograms of strain Y, even after exposure for 30 d (cf. Fig. 1*b* and *d*). A spot congruent with a fourth spot at the coordinates 74×68 (Fig. 1*d*) was present on the fluorogram of strain Y after exposure for 30 d, but was undetectable after exposure for 10 d (Fig. 1*b*). Strain 801 is the only strain of bovine origin in the LC group. It seems unlikely, although the possibility cannot be excluded, that it could have originated from the Y strain in the laboratory. It was of the LC type, at least with respect to colony size and growth rate, when first isolated from the eye of a cow which had been inoculated subcutaneously with the SC strain KH₃J (J. R. Etheridge, personal communication). The protein pattern of strain 801 was quite different from that of KH₃J.

In interpreting the protein patterns of different strains we assume that congruent spots are composed of isofunctional proteins of identical, or nearly identical, amino acid sequence. Isoelectric focusing is potentially capable of resolving proteins differing by a single charge. Isofunctional proteins with extensive amino acid sequence homology may be resolved by the two-dimensional electrophoretic method and classed as distinct and unrelated proteins, and therefore strains which are indistinguishable by DNA hybridization methods may have large differences in protein patterns. A comparison of protein 'fingerprints' is a powerful tool for distinguishing between closely related strains or between mutants of the same strain. The method is particularly well suited to study the proteins of mycoplasmas. Because of the limited size of their genome, the protein 'fingerprints' of mycoplasmas are relatively less complex and are more readily interpretable than those of other micro-organisms.

We conclude that the LC strains are more closely related to *M. mycoides* subsp. *capri* than to the SC strains of *M. mycoides* subsp. *mycoides*, and that neither the subsp. *capri* nor the LC strains are closely related to the SC strains of *M. mycoides* subsp. *mycoides*.

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