Classification of the F38 Group of Caprine Mycoplasma Strains by DNA Hybridization

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Representatives of groups of mycoplasmas which have antigens in common with *Mycoplasma mycoides* (as demonstrated by different serological tests) were compared by nucleic acid hybridization. Determinations of DNA homology were performed by filter hybridization as well as hybridization in solution; no differences were revealed between the two methods. Genetic relatedness was not demonstrated between *M. primatum* and strain F38. The antigenic similarities between strain F38 and the type strain of *M. primatum* (HRC292) may be due to common epitopes. DNA from strain F38 hybridized with DNA from *M. capricolum* (California kid) to 80%, but only to about 40% with the two *M. mycoides* subspecies, a result which can justify the classification of the F38 group as a variant of *M. capricolum*. The representative strain of bovine serogroup 7 of Leach was equally distant from F38, *M. capricolum* and the two subspecies of *M. mycoides* (approximately 60% hybridization).

INTRODUCTION

The F38-like group of mycoplasmas are aetiologically important in contagious caprine pleuropneumonia (CCPP) in Kenya. The primary analysis of F38 and a closely related strain G69 demonstrated serological identity between the two strains. Differences from *Mycoplasma mycoides* subsp. *mycoides* and *capri* were apparent, but there was some antigenic relationship as the agar gel diffusion test showed the F38 group to share at least two antigens with the type strains of the two subspecies of *M. mycoides* (MacOwan & Minette, 1976). Common antigens were also demonstrated by the growth precipitation test (Ernø & Salih, 1980). Further serological and biological characterization carried out in the laboratories at Kabete, Aarhus and Norwich (Kaliner & MacOwan, 1976; MacOwan & Minette, 1977; MacOwan & Minette, 1978; Ernø *et al.*, 1979), has demonstrated that the F38 group presents difficult taxonomic problems, since serological cross-reactions are seen in the gel double diffusion test, the complement fixation test and the growth precipitation test, with the serum digestion-positive groups of mycoplasmas (*M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri*, bovine serogroup 7 of Leach, and *M. capricolum*). Cross-reactions are seen also with *M. primatum*, a mycoplasma species isolated from primates, and *M. equigenitalium* from horses (H. Ernø & M. M. Salih, unpublished results). The F38 group of mycoplasmas differs significantly from *M. primatum* and *M. equigenitalium* in its biochemical reactions, while it is very similar to *M. mycoides*, group 7 and *M. capricolum*; it is also important to note that the immunological similarities between *M. primatum*, *M. equigenitalium* and F38 are seen only in tests depending on surface antigens, such as growth inhibition and immunofluorescence (H. Ernø & M. M. Salih, unpublished results).

The purpose of the present work was to determine whether the serological relationship between F38 and the type strain of *M. primatum* is due to demonstrable genetic relatedness, or to the presence of similar antigens in both groups, without phylogenetic significance. The possibility that the F38 group is so distinct that it should be considered a new species was also investigated.

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METHODS

Organisms, media, labelling and growth conditions. All cultures used for the experiments were delivered from the lyophilized stock cultures of the FAO/WHO Collaborating Centre for Animal Mycoplasmas. The following type and/or reference strains were used: Y-goat (*M. mycoides* subsp. *mycoides*, LC type, supplied by A. H. Dardiri) [According to Cottew & Yeats (1978), goats may harbour 2 groups of mycoplasmas classified serologically as *M. mycoides* subsp. *mycoides*. One group, the 'large colony (LC) type', does not seem to be pathogenic for cattle, whereas the other group, the 'small colony (SC) type' may be so.]; PG3 (*M. mycoides* subsp. *capri*, supplied by D. G. ff. Edward); PG50 (bovine serogroup 7 of Leach, supplied by D. G. ff. Edward); F38 (supplied by K. J. MacOwan); California kid (*M. capricolum*, from J. G. Tully); and HRC292 (*M. primatum*, ATCC 25948). In this work the strains California kid, F38, PG3 and Y-goat are referred to as the 'CCPP group'.

For DNA preparation, the mycoplasmas were grown in 5 l Bacto Heart Infusion Broth (Difco), supplemented with 16% (v/v) horse serum, 8% (v/v) of a 25% (w/v) fresh yeast extract, 0.008% (w/v) thallium acetate, and penicillin (400 i.u. ml⁻¹). *In vivo*-labelled DNA was produced by growing mycoplasmas in liquid medium containing 1.0 µCi ml⁻¹ of methyl [³H]thymidine (New England Nuclear, 6.7 Ci mmol⁻¹; 248 GBq mmol⁻¹). The cultures were incubated aerobically at 37 °C.

Isolation of DNA. This was done according to Christiansen *et al.* (1981). The cells were harvested by centrifugation at 27000 g (Sorvall, GSA rotor, 13000 r.p.m.) for 30 min at 4 °C and resuspended in 0.15 M-NaCl, 0.1 M-Na₂EDTA, pH 8.0. The micro-organisms were lysed by the addition of SDS to a concentration of 1% (w/v) followed by incubation at 60 °C for 10 min. After deproteinization by phenol and extensive dialysis against a solution of 0.3 M-NaCl, 0.01 M-EDTA, 0.01 M-Tris/HCl, pH 7.5, the nucleic acids were precipitated with ethanol. The precipitate was dissolved in a mixture containing 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0, and digested with pancreatic ribonuclease (50 units ml⁻¹) and T₁ ribonuclease (10 units ml⁻¹) for 30 min and by pronase (0.1 mg ml⁻¹) for 2 h. The DNA was banded in a caesium chloride gradient with ethidium bromide, identified by fluorescence and collected by needle puncture. The final purification was by dialysis followed by ethanol precipitation. Spectrophotometry was used to determine DNA concentrations and all preparations were analysed by analytical CsCl gradients with *Micrococcus luteus* DNA as a marker.

DNA-DNA hybridization experiments. (i) *Labelled DNA.* *In vivo* ³H-labelled DNA had specific activities of 10000 to 20000 c.p.m. µg⁻¹. *In vitro* labelling of DNA with ³²P by nick-translation (Rigby *et al.*, 1977) was to 1–3 × 10⁶ c.p.m. µg⁻¹. (ii) *Filter hybridization.* Samples (1 µg) of alkaline denatured DNA (unlabelled) were filtered through nitrocellulose filters (Millipore type HAWP, 25 mm). The buffer used for filtration was 2 M-NaCl, 0.005 M-MgCl₂, 0.05 M-Tris/HCl, pH 7.5. Filters were incubated after heat-fixation in Denhardt's medium (Denhardt, 1966) for 2 h. Hybridization was in 0.3 M-NaCl, 0.03 M-trisodium citrate, pH 7.0 with 0.5 µg ³H-labelled DNA, or 0.01 µg ³²P-labelled DNA plus 0.5 µg *Escherichia coli* carrier DNA added per filter. Filters with *E. coli* DNA reacted to background level with ³H-labelled mycoplasma DNA. (iii) *Hybridization in solution.* This was done essentially according to Aulakh & Gallo (1977) and Britten *et al.* (1974). The hybridization Mixture contained 20 µg unlabelled DNA ml⁻¹ and 0.01 µg labelled DNA ml⁻¹ in a volume of 0.5 ml 0.4 M-NaCl, 0.08 M-Tris/HCl, pH 7.5, and 0.4% (w/v) SDS. The DNA was denatured and degraded by heating to 100 °C in a dilute buffer (0.01 M-Tris/HCl, pH 7.5) before adding the salts. The reaction mixture was incubated at 60 °C for 16 h [*EC*₀*t* ~ 0.65, *EC*₀*t* ~ 0.025, *EC*₀*t* (labelled DNA) ~ 0.003]. At the end of hybridization, 0.5 ml 0.5 M-NaPB (sodium phosphate buffer) and 1 ml of H₂O was added. The DNA was analysed on a hydroxylapatite column equilibrated with 0.125 M-NaPB, 0.4% (w/v) SDS at 60 °C. ³²P passing the column at the application or at the wash with 0.15 M-NaPB was considered to be unhybridized DNA, while material eluted at 0.4 M-NaPB was taken to be hybridized DNA.

RESULTS AND DISCUSSION

G + C contents of DNA. Buoyant densities and G + C contents of the analysed DNA were calculated according to Schildkraut *et al.* (1962). The G + C contents agree with those previously reported (Askaa *et al.*, 1973). All strains have G + C contents very close to 24 mol % [F38, 24.4%; *M. mycoides* subsp. *capri*, 24.3%; *M. mycoides* subsp. *mycoides* (LC type), 23.8%; *M. capricolum*, 24.0%; *M. primatum*, 23.8%]; the representative of the bovine serogroup 7 (PG50) deviated slightly with a base composition of 25.4 mol % G + C.

Comparison of hybridization experiments using the filter technique or hybridization in solution. DNA homology was analysed by filter hybridization as well as hybridization in solution in order to compare the two methods. With either method the determined homology is the average of the two readings constituting a full hybridization cross (labelled DNAa against unlabelled DNAb and labelled DNAb against unlabelled DNAa). Only small differences were seen between the

Table 1. Results of hybridization of DNA from mycoplasmas from the CCPP group* and from *M. primatum*

Results are the average of four experiments of different types (i.e. hybridization in solution or on filter) $\pm$ S.D.

Species/serogroup	Strain	Percentage homology obtained against:					
		California kid	F38	PG50	PG3	Y-goat	HRC292
<i>M. capricolum</i>	California kid	100					
F38-like	F38	80 $\pm$ 7	100				
Bovine serogroup 7	PG50	58 $\pm$ 8	60	100			
<i>M. mycoides</i> subsp. <i>capri</i>	PG3	40 $\pm$ 6	39 $\pm$ 5	64 $\dagger$	100		
<i>M. mycoides</i> subsp. <i>mycoides</i>	Y-goat (LC type)	41 $\pm$ 8	36 $\pm$ 6	57 $\pm$ 7	75 $\pm$ 4	100	
<i>M. primatum</i>	HRC292 $\ddagger$	0.1	0.8	ND	0.2	0.3	100

ND, Not determined.

* In this work the strains California kid, F38, PG3 and Y-goat are referred to as 'the CCPP group'.

$\dagger$ Determination only with ^3H -labelled DNA in filter hybridization experiments.

$\ddagger$ Determination only with ^{32}P -labelled DNA in filter hybridization experiments.

two methods. For example, *M. mycoides* subsp. *mycoides* gave 73% homology to *M. mycoides* subsp. *capri* with hybridization analysis in solution, while the equivalent figure on filter hybridization was 75%. Similarly *M. capricolum* and *M. mycoides* subsp. *capri* gave 45% and 38% homology, F38 and *M. mycoides* subsp. *capri* 43% and 39% homology and F38 and *M. capricolum* 82% and 80% homology. Differences in results using the filter technique with high (quantitative) inputs of DNA or with low input were not observed. It was concluded, therefore, that hybridization in solution and on filters, at least in this study, give identical results.

DNA homology between the CCPP group of mycoplasmas and M. primatum. Table 1 shows the results of all hybridization experiments. No genetic relatedness was demonstrated between *M. primatum* and the CCPP group. Such a relationship would be of particular interest, since both groups occur in the same geographical area, making adaptation to a new host possible. However, it must be concluded that the serological cross-reactions noted between the type strain of *M. primatum* (HRC292) and strain F38 are attributable to similar cross-reacting antigens that do not reflect a close phylogenetic relationship.

DNA homology within the CCPP group of mycoplasmas. Strain F38 hybridized with *M. capricolum* (California kid) to 80%, a result which justifies the classification of the F38 group as a variant of *M. capricolum*. This solution of the taxonomic question would be analogous to the subspeciation of *M. mycoides* subsp. *capri* (strain PG3) and *mycoides* (strain PG1), which show 84% DNA homology (Askaa *et al.*, 1978). The two subspecies of *M. mycoides* are, like *M. capricolum* and F38, serologically distinct, when methods usually considered species specific (immunofluorescence, growth inhibition, and metabolism inhibition) are used, but it is recognized in this laboratory that some field strains are isolated, which react with antisera against both subspecies. Similarly, *M. capricolum* and the F38 group may occasionally cross-react in immunofluorescence and growth inhibition, but they differ more markedly in their biochemical properties than is the case with the subspecies of *M. mycoides*.

Strain F38 is apparently closer to *M. capricolum* (80% homology) than the two defined subspecies of *M. mycoides* [subsp. *capri* (PG3) and subsp. *mycoides* (LC type, Y-goat)] are to each other with 75% homology. Askaa *et al.* (1973) investigated the relationships within the CCPP group by DNA/DNA hybridizations. The results presented here are, in the few comparable cases, in agreement with those of Askaa *et al.* (1973) taking into consideration their rather large standard deviations.

Using the terminology of Johnson (1973) we can conclude that the CCPP group of mycoplasmas form an entity at a level of closely related genospecies with two clear examples at

the genosubspecies level. In this respect the classification of the two subspecies of *M. mycoides* analysed by biochemical and immunological methods is also in complete agreement with the classification based on our DNA homology analysis using the proposal of Johnson (1973). By analogy, classification of F38 as a subspecies of *M. capricolum* would appear reasonable on the basis of the DNA/DNA homology data, but definitive classification must include evaluation of biochemical and immunological properties.

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