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THE F38-LIKE GROUP, A NEW GROUP OF CAPRINE MYCOPLASMAS?

By

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ERNØ, H., R. H. LEACH, M. M. SALIH, and K. J. MACOWAN: *The F38-like group, a new group of caprine mycoplasmas?* Acta vet. scand. 1983, *24*, 275—286. — This paper concerns the taxonomic status of the F38-like group (*MacOwan*), a prime determinant of contagious caprine pleuropneumonia (CCPP). Extensive biochemical and serological investigations on strain F38 are reported. Some complex serological relationships with other mycoplasma species are revealed. The results, taken in conjunction with earlier published work on genotypic characters, lead to the conclusion that final classification of these organisms should await further comparative studies of a number of field strains with a related group of strains classified as M. capricolum.

The characterization of F38 confirms its partial relationship to the "M. mycoides group" of ovine/caprine/bovine mycoplasmas, and has also revealed a very close phenotypic relationship to the bovine mycoplasma serogroup 7, a finding of potential diagnostic and epidemiological importance.

mycoplasmas; classification; F38-like group.

Isolations of biochemically and serologically identical mycoplasmas have been made from cases of contagious caprine pleuropneumonia (CCPP) in Kenya (*MacOwan* 1976, *MacOwan & Minette* 1976 and 1977). The organism is an important determinant of CCPP, a clinically acute and rapidly spreading disease (*McMartin et al.* 1980). It has also been isolated recently from cases of the same disease in the Sudan (*Harbi et al.* 1981), and

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North Africa (*Perreau* 1981). Partial characterization of the Kenyan strain, F38, suggested that the F38-like group of mycoplasmas might represent a new species (*Ernø et al.* 1979). However, further examinations revealed unusually complicated serological cross-reactions between F38 and several established species. This situation required further investigation, and the present paper represents the results of a collaborative study between the FAO/WHO Collaborating Centre for Animal Mycoplasmas (AMRC) and the Mycoplasma Reference Laboratory (MRL), to determine the appropriate taxonomic status of the F38-like group.

MATERIALS AND METHODS

Mycoplasma strains

Strain F38 from lesions of acute CCPP (*MacOwan & Minette* 1976) was purified by the triple-filter cloning method (*Subcommittee* 1979). The 69 strains (Table 1), were from the culture collections of AMRC or MRL.

Media

At MRL, the fluid medium used for cultivation of F38 was that of *Hayflick* (1965), supplemented for various tests, as described elsewhere (*Leach* 1973). The solid media were Hayflick agar, MRL agar (*Gourlay et al.* 1974) and LH agar (*Allam & Lemcke* 1975).

At AMRC, strain F38 was cultivated in a modified Hayflick medium (B) (*Ernø & Stipkovits* 1973 a). Other mycoplasmas (Table 1) used for serological comparison were cultivated in media suitable for each species (*Freundt et al.* 1979).

Morphology

Colonies were examined by light microscopy at × 25 periodically during incubation for 10 days at 37°C in an atmosphere of 5 % CO_2 and 95 % N_2.

Cells from broth cultures were examined by phase contrast, darkfield, and interference contrast microscopy.

For electron microscopy, the procedure for fixation of colonies, sectioning and staining was that of *Vinther* (1976).

Table 1. Type or reference* strains of 66 currently recognized Mycoplasma species.

Species	Strain	Species or serogroup	Strain
M. agalactiae	PG2	M. gallinarum	PG16
M. alkalescens	PG51 (D12)	M. gallisepticum	PG31
M. alvi	Ilsley	M. gallopavonis	WR1
M. anatis	1340	M. gateae	CS
M. arginini	G230	M. hominis	PG21
M. arthritidis	PG6	M. hyopneumoniae	J
M. bovigenitalium	PG11	M. hyorhinis	BTS-7
M. bovirhinis	PG43	M. hyosynoviae	S16
M. bovis	Donetta	M. iners	PG30
M. bovoculi	M165/69	M. iowae	695
M. buccale	CH20247	M. lipophilum	MaBy
M. californicum	ST-6	M. maculosum	PG15
M. canadense	275C	M. meleagridis	17529
M. canis	PG14	M. moatsii	MK405
M. capricolum	California kid	M. molare	H542
M. caviae	G122	M. mustelae	MX9
M. citelli	RG-2C	M. mycoides subsp. capri	PG3
M. columbinasale	694	M. mycoides subsp. mycoides	PG1
M. columbinum	MMP-1	M. mycoides subsp. mycoides	("LC type") Y-goat
M. columborale	MMP-4	M. neurolyticum	Type A
M. conjunctivae	HRC581	M. opalescens	MH5408
M. criceteli	CH	M. orale	CH19299
M. cynos	H831	M. ovipneumoniae	Y-98
M. dispar	462/2	M. pneumoniae	FH
M. edwardii	PG24	M. primatum	HRC292
M. equigenitalium	T37	M. pullorum	CKK
M. equirhinis	M432/72	M. pulmonis	PG34 (Ash)
M. fastidiosum	4822	M. putrefaciens	KS-1
M. faucium	DC-333	M. salivarium	PG20
M. feliminutum	BEN	M. spumans	PG13
M. felis	CO	M. sualvi	Mayfield
M. fermentans	PG18	M. subdolum	TB
M. flocculare	Ms42	M. synoviae	WVU1853
M. gallinaceum	DD	M. verecundum	107

* The representative strain of bovine group 7, PG50 (N29) was included in the study.

Filterability

The filterability of F38 was determined by using membrane filters (Millipore Corporation) with pore diameters of 450, 220, and 100 nm. Estimation of the number of colony-forming units per ml (cfu/ml) was carried out before and after filtration of 2-day-old broth cultures.

Reversion

In order to assess whether reversion to bacterial forms could occur, the organisms were passaged serially 5 times in standard broth (B), without inhibitors, and given one further, final passage on standard agar, also without inhibitors.

Cholesterol requirements

Requirement for cholesterol was determined on solid medium by the method of *Edward* (1971). Three different media were used: (i) medium CA, a basal medium consisting of heart infusion agar (Difco), 90 ml; DNA (0.2 % w/v solution; Sigma), 1—2 ml; benzylpenicillin (20,000 IU/ml), 0.25 ml; thallium acetate (1 % w/v solution), 1 ml; (ii) medium CB, the same as CA, but supplemented with final concentrations of palmitic acid (0.1 % w/v), and bovine serum albumin (5 % w/v); (iii) medium CC, the same as CB but supplemented with cholesterol (0.05 % w/v, final concentration). A twice-washed suspension of F38, obtained from a culture grown in 200 ml of standard medium (except that horse serum had been replaced by Difco PPLO serum fraction, 1 % v/v), was decimally diluted in PBS from 10^0 to 10^{-6}. From each dilution, 0.01 ml was streaked on the 3 different media (CA, CB, and CC). The inoculated plates were incubated at 37°C in air with 8 % CO_2. Sterol requirement was also checked indirectly by sensitivity to digitonin (*Ernø & Stipkovits* 1973 a).

Biochemical characteristics

Tests for fermentation of glucose, hydrolysis of urea and of arginine, phosphatase activity, serum digestion, reduction of triphenyltetrazolium chloride, and formation of "film and spots" on egg-yolk medium were performed as described previously (*Ernø & Stipkovits* 1973 b).

Serological tests

Hyperimmune rabbit sera against 69 type and reference strains (Table 1) were prepared at AMRC (*Ernø et al.* 1973). Some used at MRL were prepared there by a slightly different method (*Langford & Leach* 1973).*

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* Goat antiserum against M. sualvi was kindly supplied by *R. N. Gourlay* and rabbit antiserum to M. lipofaciens by *Janet Bradbury*.

T a b l e 2. Serological tests showing the cross-reactions between the F38-like group and other mycoplasma species and bovine serogroup 7.

Species or serogroup	Strain	Antiserum																							
		IMFT								GIT								GPT							
		F38	PG 50	Cal. kid	PG1	Y-goat	PG3	T37	HRC 292	F38	PG 50	Cal. kid	PG1	Y-goat	PG3	T37	HRC 292	F38	PG 50	Cal. kid	PG1	Y-goat	PG3	T37	HRC 292
F38-like	F38	+	+	0	0	0	0	+	+	+	+	0	0	0	0	+	+	+	+	+	+	+	+	0	0
Bovine serogroup 7	PG50	+	+	0	0	0	0	+	+	+	+	0	+	0	+	+	+	+	+	+	+	+	+	0	0
M. capri-colum	Calif. kid	0	0	+	0	0	0	0	0	0	0	+	0	0	0	0	0	+	+	+	+	+	+	0	0
M. mycoides subsp. myc.	PG1	0	0	0	+	+	0	0	0	0	0	0	+	+	0	0	0	+	+	+	+	+	+	0	0
M. mycoides subsp. myc.	Y-goat	0	0	0	+	+	0	0	0	0	0	0	+	+	0	0	0	+	+	+	+	+	+	0	0
M. mycoides subsp. capri	PG3	0	0	0	0	0	+	0	0	0	0	0	0	0	+	0	0	+	+	+	+	+	+	0	0
M. equigeni-talium	T37	+	+	0	0	0	0	+	+	+	+	0	0	0	0	+	+	0	0	0	0	0	0	+	0
M. primatum	HRC292	+	+	0	0	0	0	+	+	+	+	0	0	0	0	+	+	0	0	0	0	0	0	0	+

IMFT: Indirect immunofluorescence test
GIT: Growth inhibition test
GPT: Growth precipitation test
+: Positive
0: Negative

Strain F38 was tested with these antisera by the agar well modification of the growth inhibition (GI) technique (*Black* 1973), the indirect immunofluorescence (IMF) technique (*Rosendal & Black* 1972), and the growth precipitation (GP) technique (*Ernø & Peterslund* 1983). Hyperimmune serum against F38 was tested by each method against all 69 strains. Sera from 2 goats, one from a field case of CCPP and one from a goat immunized with F38, were supplied by British Overseas Development Project R2820 from the Veterinary Research Laboratories, Kabete, Kenya. These sera were tested in GI tests against F38 and M. primatum only.

RESULTS

Cultural and morphological characteristics

Strain F38 was able to produce colonies, having the classical "fried egg" appearance, after incubation for 3 days at 37°C in 5 % CO_2 and 95 % N_2 or in a candle jar. Aerobic growth was slower, colonies appearing only after 5 days. Maximum colony size (0.5 mm) was obtained after candle jar incubation for 7 days. Growth in liquid medium reached a maximum after 7 days aerobic incubation. No growth was observed at 22°C. Microscopy of broth cultures after 7 days' incubation showed pleomorphic organisms (coccoid, ring, and filamentous forms, sometimes with a stellate appearance). Branching filaments were occasionally seen, up to 3 μm in length. Electron microscopy revealed pleomorphic cells surrounded by the three-layered membrane typical of mycoplasmas, and without any cell wall.

Filterability

After filtration of an F38 culture, containing 10^9 cfu/ml, through membrane filters of 450, 220, and 100 nm pore diameters, the counts were 10^5, 10^3, and $< 10^2$ per ml, respectively.

Reversion

Reversion to bacterial colony types did not occur.

Cholesterol requirements

The washed suspension of F38, containing 10^8 cfu/ml as tested on standard medium B, produced no growth on the basal sterol-free agar medium (CA), nor on the same medium with

palmitic acid and bovine serum albumin (CB). On the medium further supplemented with cholesterol (CC), growth occurred only on plates inoculated with 10°, 10^{-1}, 10^{-2}, and 10^{-3} dilutions (i.e. inocula of $\geq 10^3$ cfu). F38 was sensitive to digitonin.

Biochemical characteristics

Strain F38 fermented glucose aerobically and anaerobically, and reduced tetrazolium chloride anaerobically, but not aerobically. Hydrolysis of urea and arginine, phosphatase activity, and formation of "film and spots" were not detected. The serum digestion test gave a clearcut positive result provided the tubes were incubated in 5 % CO_2 and 95 % N_2.

Serological characteristics

The two-way serological comparison of F38 with 66 previously-established Mycoplasma species as well as bovine serogroup 7 of *Leach* (1973) revealed a number of cross-reactions of different types by IMF, GI, and GP tests. (I) Strain F38 showed one-way cross-reactions with antiserum against M. bovigenitalium in GI and IMF tests, and a one-way cross-reaction in the GI test against M. alvi antiserum. (II) Two-way cross-reactions were observed with M. equigenitalium and M. primatum, but only in IMF and GI tests (Table 2). M. primatum was also inhibited by the goat serum from a clinical case and the goat hyperimmune serum. (III) Cross-reactions occurred consistently in the GP test between F38 and the reference strains for M. putrefaciens, M. capricolum, M. mycoides subsp. capri, M. mycoides subsp. mycoides (PGl), and Y-goat, the reference strain of the LC (large-colony) type of M. mycoides subsp. mycoides (*Cottew & Yeats* 1978). (IV) Two-way cross-reactions between F38 and serogroup 7 were observed with all three serological tests (Table 2).

Apart from these notable cross-reactions, F38 was serologically distinct from all other Mycoplasma species studied.

DISCUSSION

The above results together with published information provide a description of strain F38. The lack of cell wall, cellular morphology, filterability through a membrane filter of pore diameter 450 nm, colonial morphology and failure to revert to a bacterial form place F38 in the class Mollicutes, order Myco-

plasmatales. Its base composition (G+C) of 24.4 % (*Christiansen & Ernø* 1982) conforms with this classification. The sensitivity to digitonin, requirement of cholesterol for growth, lack of spiral morphology, and inability to hydrolyze urea further classify F38 within the family Mycoplasmataceae and genus Mycoplasma.

The delineation of species within the genus Mycoplasma has traditionally rested on mainly serological criteria. Most earlier mycoplasma species were serologically distinct, but interspecies cross-reactivity has become more frequent with increasing numbers of species, making serology less reliable for classification.

The taxonomy of F38 represents a prime example of complicated serological relationships among mycoplasmas. This strain having been compared with reference strains for 67 named Mycoplasma species including bovine serogroup 7, showed varying degrees of serological cross-reactivity with several of them, most notably with M. capricolum, M. mycoides subspp. mycoides and capri, M. equigenitalium, M. primatum, and serogroup 7. Before evaluation of these relationships, some discussion of the taxonomic significance of the various serological methods involved is necessary.

Antigens of mycoplasmas can be divided mainly into internal (cytoplasmic) and surface antigens. The surface antigens may be membrane-bound or part of some extra-membraneous components. Of the serological tests employed, GI and IMF are best suited to detect surface antigens, and these are usually species-specific. However, common surface antigens between different Acholeplasma species have been detected with these tests (*Ernø & Salih* 1980). Furthermore, in our experience older cultures (especially of M. mycoides) may give negative homologous reactions in these two tests, perhaps due to accumulating extramembraneous material obscuring membrane-bound antigens. Thus the methods most used for demonstrating shared surface antigens may show false positive as well as false negative results in identification at the species level.

The GP test detects and resolves soluble cytoplasmic and soluble extramembraneous antigens. Although seemingly well-suited to detect cytoplasmic antigens shared between species (*Ernø & Salih* 1980), it is also rather insensitive, like all precipitation-based tests, and prone to give false negative reactions (*Ernø & Peterslund* 1983).

Our tests produced various degrees of serological cross-reac-

tivity between F38 and other mycoplasmas. Two of these (categories (I) and (II) — "Results") were revealed only by GI and/or IMF tests. Those involving M. alvi and M. bovigenitalium showed only one-way reactions and are of dubious taxonomic significance. The two-way reactions shown by F38 with M. equigenitalium and M. primatum demand greater consideration, but even these cannot be taken to indicate species relationships, since the GP tests revealed no shared antigens. This negative inference is supported by the discrepant cultural and biochemical properties of the cross-reacting species, and, in specific instances, by published results of hybridization experiments showing no homology at all between F38 and M. primatum (*Christiansen & Ernø* 1982), and isoenzyme analyses clearly differentiating F38 from M. primatum, M. bovigenitalium, and M. equigenitalium (*Salih et al.* 1983).

For M. mycoides subsp. mycoides, M. mycoides subsp. capri, M. capricolum, and M. putrefaciens, serological relationships with F38 were demonstrable only by the GP test (category (III) — "Results"). The failure of GI and IMF tests to confirm these cross-reactions probably indicates taxonomic relationships with F38 more distant than at species level. For the two M. mycoides subspp., this conclusion is clearly endorsed by the low level of homology (40 %) shown in DNA hybridization experiments (*Christiansen & Ernø* 1982), and also in their high dissimilarity coefficients (0.33 and 0.44, respectively) obtained from isoenzyme analyses (*Salih et al.* 1983). For M. capricolum the position is somewhat more complicated, in that DNA hybridization test results (*Christiansen & Ernø* 1982) do indicate a relatively high level (80 %) of genetic homology with F38, whereas the indirect genetic evidence from iso-enzyme analysis tends to emphasize their species differences, with a dissimilarity coefficient of 0.30, higher than for two such clearly distinct species as M. ovipneumoniae and M. capricolum (*Salih et al.* 1983). However, in our experience some field strains of M. capricolum and the F38-like group do cross-react in GI and IMF tests. Therefore, it seems necessary to compare a greater number of strains of these groups, especially by DNA/DNA hybridization, isoenzyme-analysis, and 2D-electrophoresis.

The closest serological relationship (category (IV) — "Results") with F38 was shown by bovine serogroup 7 (strain PG50). None of the three serological tests used was able to separate

them, nor were additional metabolism inhibition tests (*H. Ernø* and *R. H. Leach*, unpublished results). However, in previous DNA hybridization studies (*Christiansen & Ernø* 1982), PG50 was equally distinct from F38 as from M. capricolum and either subsp. of M. mycoides (each ca. 60 % hybridization). Moreover, isoenzyme analyses (*Salih et al.* 1983) indicated a dissimilarity coefficient for F38 and PG50 that is relatively high (0.34), a further pointer to their separateness as species. For the present taxonomic purpose, the relationship of F38 to an unnamed serogroup is largely irrelevant. However, veterinarians should note that these bovine and caprine organisms cannot yet be readily distinguished by routine diagnostic methods.

From the present and previously published data discussed above it is concluded that the taxonomic — and pathogenic — relationship between M. capricolum, bovine group 7, and the F38-like group should be further examined before final classification can be made.

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SAMMENDRAG

Gruppe F38, en ny gruppe af mykoplasmer isoleret fra geder?

Artiklen omhandler den taksonomiske placering af den caprine gruppe F38 (*MacOwan*), en vigtig determinant for smitsom, ondartet lungesyge hos geder (CCPP). Omfattende biokemiske og serologiske undersøgelser af stamme F38 rapporteres. Der findes komplicerede, serologiske relationer til andre arter af mykoplasmer. Resultaterne, sammenstillet med tidligere publicerede arbejder af genetisk karakter, fører til den konklusion, at den endelige klassification af disse mikroorganismer bør afvente udvidede sammenlignende studier af et større antal stammer med en relateret gruppe, klassificeret som M. capricolum.

Undersøgelserne af F38 bekræfter dens partielle relation til „M. mycoides gruppen“ af ovine, caprine og bovine mykoplasmer. Der ses endvidere et meget nært phænotypisk slægtskab til bovin gruppe 7, hvilket er af potentiel diagnostisk og epidemiologisk betydning.

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