Biochemical diversity within the '*Mycoplasma mycoides* cluster'

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The metabolism of 51 strains within the '*Mycoplasma mycoides* cluster' was investigated by measuring oxygen uptake following the addition of organic substrates to washed cell suspensions. There were extensive differences between strains in the range of substrates utilized, the relative rates of oxidation and the observed saturation constants for substrates, which ranged from a few μM to several mM. *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC (large colony) strains were diverse and could not be distinguished by substrate utilization patterns. However, there were consistent differences in the patterns of substrate utilization between other groups of the *M. mycoides* cluster, suggesting that these patterns may be useful in identification. In particular, SC (small colony) strains of *M. mycoides* subsp. *mycoides* were distinguished by their inability to oxidize maltose, trehalose and (at low concentrations) mannose and glucosamine. Surprisingly, the type strain, *M. F38*, of *Mycoplasma capricolum* subsp. *capripneumoniae* and two further isolates differed from all other strains in that they did not oxidize glucose or other sugars. They did, however, oxidize pyruvate, lactate and 2-oxobutyrate at high rates. The marked metabolic differences between these strains and *M. capricolum* subsp. *capricolum* strains is in contrast to the genetic evidence that was used to support the designation of the *M. F38* group as a subspecies of *M. capricolum*.

Keywords: *Mycoplasma mycoides* cluster, *Mycoplasma capricolum*, substrate oxidation patterns, mycoplasma identification

INTRODUCTION

The genus *Mycoplasma* currently contains more than 80 recognized species. These may be categorized according to whether they produce acid from glucose metabolism, ammonia from arginine hydrolysis, or both or neither of these reactions. A few additional, readily determined biochemical tests (Freundt & Razin, 1984) may also be useful as a guide to identification; however, routine serological tests are always required to determine species or subspecies identity. Furthermore, the absence of

specific serological reactivity with established species is currently the main criterion for recognition of new species (Anon., 1979).

The dependence upon serological tests for *Mycoplasma* characterization can lead to difficulties in the identification of new isolates if there are extensive serological cross-reactions between species or subspecific groups. Such difficulties are particularly evident within the groups of strains referred to by Cottew *et al.* (1987) as the '*Mycoplasma mycoides* cluster' (Cottew, 1979; Cottew *et al.*, 1987; Nascimento *et al.*, 1986; Salih *et al.*, 1983). This cluster comprises six groups with the following original designations: *M. mycoides* subsp. *capri*; *M. mycoides* subsp. *mycoides* small colony (SC) strains; *M. mycoides* subsp. *mycoides* large colony (LC) strains; Bovine serogroup 7; *M. capricolum*; and the F38-like group. The latter two have now been designated separate subspecies of *M. capricolum* and named subsp. *capricolum* and *capripneumoniae*, respectively (Leach *et al.*, 1993). All groups are pathogens and the

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Abbreviations: d.o.t., dissolved oxygen tension; GlcNAc, N-acetylglucosamine; PEP:PTS, phosphoenolpyruvate:phosphotransferase; SC, small colony; LC, large colony.

principal hosts are cattle, goats or sheep. The SC strains of *M. mycoides* subsp. *mycoides* cause contagious bovine pleuropneumonia (CBPP), a disease of particular economic importance which is widespread in Africa, Asia and the Middle East, and a cause of concern in Europe following recent outbreaks in France, Portugal, Spain and Italy (ter Laak, 1992). The majority of SC strains have been isolated from cattle, but isolations have also been made from goats (see Cottew, 1979). The LC biotype of *M. mycoides* subsp. *mycoides* may also occur in both cattle and goats and unclassified subsp. *mycoides* strains have been reported in sheep (Okoh & Ocholi, 1986; Cottew, 1979). *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae* are mainly associated with goats, the latter being the causative agent of classical contagious caprine pleuropneumonia (CCPP; MacOwan & Minette, 1977).

Despite clear cultural and other phenotypic differences, there is a particularly close serological relationship between the *M. mycoides* subsp. *mycoides* SC and LC strains and many of the dominant protein antigens are shared (Archer, 1979). Some of these are also shared by subsp. *capri* strains and analysis of cell proteins by gel electrophoresis suggests that LC strains of *M. mycoides* subsp. *mycoides* are more closely related to *M. mycoides* subsp. *capri* than to SC strains (Costas *et al.*, 1987; Leach *et al.*, 1989; Rodwell & Rodwell, 1978). *M. capricolum* subsp. *capricolum* and *capripneumoniae* are also closely related on these grounds, as befits their subspecies classification. In addition, DNA-DNA hybridization was from 82 to 100% amongst subsp. *capricolum* strains, 87 to 100% amongst *M. F38*-related strains and 67 to 73% between members of the two groups under high-stringency conditions (Anon., 1991; Bonnet *et al.*, 1993). These findings led to the designation of these two groups as subspecies within *M. capricolum* (Leach *et al.*, 1993).

The development of easily determined biochemical testing procedures for mycoplasmas could help overcome the problem of serological cross-reaction and simplify current identification procedures. However, the development of such procedures is less well advanced than for other bacterial groups. In particular, substrate utilization tests, of great value in the characterization of other bacteria, are little used for mycoplasmas, except those for glucose and arginine. The routine detection of substrate metabolism during mycoplasma growth is often not feasible because of the low concentrations of organisms, and because of difficulties in detecting the metabolism of specific substrates in the presence of the complex medium components needed to support mycoplasma growth which provide a vast array of alternative substrates. In addition, the utilization of di- and polysaccharides of glucose cannot be determined in serum-containing medium, since they are rapidly hydrolysed by serum enzymes (Miles & Lee, 1983).

The metabolism of specific substrates by cells suspended in an inorganic salts solution has been monitored using microcalorimetry for *M. mycoides* subsp. *mycoides* strain T1 (Miles *et al.*, 1985), and by measuring changes in the

dissolved oxygen tension of cell suspensions for *M. mycoides* subsp. *capri* strain PG3 (NCTC 10137; Miles & Wadher, 1990). In this paper, we report the application of the latter technique to the determination of substrate utilization by 51 strains representing the six groups of the *M. mycoides* cluster. The substrates tested in this study included all those previously shown to be metabolized by *M. mycoides* subsp. *mycoides* strain T1 and other bovine, caprine and ovine mycoplasmas (Wadher *et al.*, 1990), except that threonine and serine (utilized at low rates by strain T1) were excluded and 2-oxobutyrate, a likely intermediate in threonine metabolism (Rodwell, 1960), was included.

A major aim of this work was to determine whether patterns of substrate utilization could distinguish the members of the *M. mycoides* cluster. However, the technique also provides kinetic data allowing assessment of the likely significance of substrate metabolism at the concentrations found *in vivo*. Differences in substrate utilization patterns between isolates may be related to host or tissue specificity and thus aid understanding of pathogenicity.

METHODS

Organisms. The strains used are listed in Table 1. They were obtained as broth cultures or freeze-dried ampoules and upon receipt were subcultured in broth, dispensed in ampoules and stored in liquid nitrogen for further use. For brevity, *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae* (F38-type) will be referred to throughout this paper simply as 'subsp. *capricolum*' and 'subsp. *capripneumoniae*', respectively.

Media. All mycoplasmas except subsp. *capripneumoniae* strains were grown in a medium containing heat-inactivated pig serum, tryptose, yeast extract, glucose, glycerol and inorganic salts, as previously described (Wadher & Miles, 1988). Subsp. *capripneumoniae* strains were grown in a medium containing proteose peptone, 15 g l⁻¹ (Oxoid); neutralized liver digest, 2.5 g l⁻¹ (Oxoid); yeast extract, 5 g l⁻¹ (Oxoid); NaCl, 5 g l⁻¹; glucose, 5 g l⁻¹; sodium pyruvate 5 g l⁻¹; fresh yeast extract (Freundt, 1983), 20 ml l⁻¹; and heat-inactivated pig serum, 200 ml l⁻¹ (Gibco). The pH value of both media was 7.6.

Growth and preparation of cell suspensions. Broth medium was dispensed in 10 ml quantities in plastic screw-capped test-tubes (Sterilin) and inoculated with 0.1 ml of a liquid nitrogen stored culture. After 48 h incubation at 37 °C, 0.1 ml quantities of these cultures were transferred to fresh medium and similarly incubated. Cells were then harvested by centrifugation at 14000 g for 4 min (MSE Microcentaur) and the cell pellets were washed and resuspended in a solution containing 18 g HEPES l⁻¹ and 160 U catalase ml⁻¹ (Sigma) in one-quarter strength Ringer's solution (Oxoid). The pH of the suspending medium was 7.6. The cell protein content of mycoplasma suspensions was determined by the Lowry method, and viable counts performed as described by Miles *et al.* (1985).

Substrate metabolism. Substrate oxidation was determined from changes in dissolved oxygen tension (d.o.t.) with an oxygen electrode system (Rank Brothers) linked to a chart recorder and calibrated with air-saturated water (Miles *et al.*, 1991). Cell suspensions (1 ml) containing 100–300 µg cell protein and a viable count of 5 × 10⁸–2 × 10⁹ c.f.u. were added

Table 1. *Mycoplasma* strains used and their sources

Strain designation*	Country of isolation
Bovine serogroup 7	
PG50 ^R (NCTC 10133)	Australia
L2917, QR1	Australia†
<i>M. capricolum</i> subsp. <i>capripneumoniae</i>	
F38 ^T (NCTC 10192), G183	Kenya†
G94/83, G1943/80	Kenya‡
<i>M. capricolum</i> subsp. <i>capricolum</i>	
California kid ^T (NCTC 10154)	USA†
607/79, 4161/88	France§
YP, YO, ZT	Australia†
PP goat	Turkey†
<i>M. mycoides</i> subsp. <i>capri</i>	
PG3 ^T (NCTC 10137)	Turkey
N108	Nigeria†
YC, ZZ, 74.5907A, JM	Australia†
Pendik, BQT	Turkey†
G108/A2 clone(a), G108/A2 clone(b)	Brazil†
G105/A1, G169/Leite, G108/A3	
<i>M. mycoides</i> subsp. <i>mycoides</i> (LC)	
Y-goat ^R , VRI, 74/2488, 81.636.1C	Australia†
GE.6A.79E, KHI, 78/441, 11041	
F30	Kenya†
OJO1	Nigeria†
COV2	Turkey†
GM12	USA†
977/79, 400/79, 755/80, 221/82	France§
1645/82, 1729/82, 842/86	
<i>M. mycoides</i> subsp. <i>mycoides</i> (SC)	
PG1 ^T (NCTC 10114)	
Gladysdale	Australia†
Oremit	Kenya†
T1	Kenya
KH3J	Sudan†

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

† See Costas *et al.* (1987); Leach *et al.* (1989).

‡ Culture provided by Professor F. R. Rurangirwa and Dr A. Kebor, Nairobi, Kenya.

§ Culture provided by Mr Maurice Lambert, CNEVA, Laboratoire de Pathologie des Petits Ruminants, 06000 Nice, France.

|| Culture provided by Dr G. R. Smith, Institute of Zoology, Regent's Park, London, UK.

to the electrode vessel which was magnetically stirred and maintained at 37 °C. The air-saturated suspension (d.o.t. approximately 210 nmol ml⁻¹) was then isolated from the air and a test substrate was added by microsyringe. Organic acids were added as their sodium salts. All experiments were repeated; the data shown are mean values of two or more replicate experiments.

Calculation of kinetic parameters of substrate utilization. In general, saturation constants (K_m values) were estimated from

recorder tracings of change in d.o.t. with time following addition of a low concentration of substrate (up to 50 µM). It was assumed that Michaelis kinetics were observed (Miles *et al.*, 1985) and that the reduction in d.o.t. at any time (t) after substrate addition was proportional to the substrate used. Thus, substrate concentration (s) at time t was (initial substrate concentration) $\times (a - b)/a$, where a was the total reduction in d.o.t. following substrate addition and b the reduction in d.o.t. at time t . The straight line of best fit was then drawn in plots of $1/(\text{rate of oxygen uptake})$ versus $1/(s)$, and the intercept on the $1/s$ axis (equal to $-1/K_m$) determined. In those cases where the affinity for substrate was low (i.e. $K_m > 50$ µM), rates of oxygen uptake were determined at a number of different initial substrate concentrations.

RESULTS

Effect of substrate concentration on rate of metabolism

A representative curve showing the change in d.o.t. after the addition of metabolizable substrate to a mycoplasma cell suspension is given in Fig. 1. No oxygen uptake (reduction in d.o.t.) was observed in the absence of substrate.

All strains, except three subsp. *capripneumoniae* strains (F38, G1943/80, G94/83), oxidized glucose (25 µM) at a high initial rate [oxygen uptake rate 40–200 nmol O₂ min⁻¹ (mg cell protein⁻¹)], which was not increased by increasing glucose concentrations tenfold; total oxygen uptake [approximately 2 mol (mol glucose)⁻¹] was consistent with the expected oxidation of added glucose to acetate plus CO₂ (Rodwell & Mitchell, 1979). K_m values for glucose oxidation were estimated to be < 5 µM and similarly low K_m values were obtained for many mycoplasma–substrate combinations (as exemplified in the results for three individual strains shown in

Fig. 1. Changes in d.o.t. following addition of oxidizable substrates to a cell suspension of *M. capricolum* strain PP goat. The substrates added were (A) glucose, (B) GlcNAc and (C) glucosamine. All substrates were added to an initial concentration of 25 nmol ml⁻¹.

Table 2. Values of K_m for utilization of substrates by representative mycoplasma strains

Substrate	K_m value (μM)		
	<i>M. mycoides</i> subsp. <i>mycoides</i> PG1	<i>M. mycoides</i> subsp. <i>capri</i> PG3	<i>M. capricolum</i> subsp. <i>capripneumoniae</i> G94/83
Glucose	3.5	4.8	ND
Maltose	ND	1.3	ND
Trehalose	ND	2.0	ND
GlcNAc	1.0	1.0	ND
Glucosamine	620	10	ND
Mannose	9.1 mM	2.8	ND
Fructose	3.9	1.7	ND
Glycerol	3.5	6.6	ND
Pyruvate	6.0	18	5.8
Lactate	260	630	4.6
2-Oxobutyrate	9.3	> 1 mM	9.7

ND, Metabolism not detected.

Fig. 2. Effect of (a) mannose and (b) L-lactate concentration on the rate of oxygen uptake by *M. mycoides* subsp. *mycoides* PG1 (NCTC 10114). The rate of oxygen uptake by cell suspensions with glucose (25 μM) was 70 nmol min⁻¹ (mg cell protein)⁻¹.

Table 2). However, in some cases apparent K_m values were high (Table 2) and substrate metabolism was detectable only at relatively high substrate concentrations (e.g. mannose utilization by *M. mycoides* subsp. *mycoides*

PG1; Fig. 2a). Also, lactate oxidation was generally detectable at low concentrations, but the rate of oxidation increased with increasing substrate concentration (e.g. *M. mycoides* subsp. *mycoides* PG1; Fig. 2b).

Comparison of rates of substrate utilization by mycoplasmas

To compare substrate utilization by different mycoplasma strains, rates of oxidation were initially determined at concentrations of 12 μM for disaccharides, 25 μM for monosaccharides and amino sugars, and 50 μM for organic acids. These low initial substrate concentrations were chosen so that, after oxygen uptake had ceased due to exhaustion of rapidly metabolized substrate, further (different) substrate could be added (Fig. 1). Substrate concentration was varied in the above manner so that total oxygen uptake would be similar for each substrate and sufficient to allow accurate determination of the initial uptake rate. Where substrate utilization was not detected at these initial concentrations of 12–50 μM , substrate concentration was increased tenfold. Rates of substrate utilization are generally compared in terms of the rate of oxygen uptake, expressed as a percentage of the rate with glucose (25 μM ; Tables 3–5). The rate for glucose was determined prior to the addition of other substrates (Fig. 1) and after utilization was complete. In replicate experiments, rates of substrate utilization were consistent.

***M. mycoides* strains.** The five *M. mycoides* subsp. *mycoides* SC strains appeared similar in the pattern and rates of substrate utilization (Table 3). All strains were unable to metabolize the disaccharides maltose and trehalose, but oxidized N-acetylglucosamine (GlcNAc), glycerol and 2-oxobutyrate at high rates. The Gladysdale strain oxidized pyruvate and lactate at rates approximately two- to fourfold higher than the other strains. In contrast, the 19

Table 3. Rate of substrate utilization by *M. mycoides* subsp. *mycoides* SC, *M. capricolum* subsp. *capricolum* and Bovine serogroup 7 strains

Rates determined at 12 μ M for disaccharide sugars, 25 μ M for monosaccharide sugars and 50 μ M for other substrates. Rates of O₂ uptake are expressed as a percentage of the initial rate for glucose; O₂ uptake rates for glucose were between 15 and 30 nmol min⁻¹ ml⁻¹. +, Oxygen uptake only detectable when substrate concentration increased tenfold; ND, oxygen uptake not detectable even when substrate concentration increased tenfold.

Substrate	Initial rate of O ₂ uptake (percentage of initial rate for 25 μ M glucose)						
	<i>M. mycoides</i> subsp. <i>mycoides</i> SC (5 strains)	<i>M. capricolum</i> subsp. <i>capricolum</i> strains			Bovine serogroup 7		
		California kid	4161/88	Others (5 strains)	PG50	QR1	L2917
Maltose	ND	28	34	15–26*	46	43	18
Trehalose	ND	ND	ND	ND	120	57	63
GlcNAc	48–72	63	12	19–38†	52	13	ND
Glucosamine	+	6	+	4–8‡	+	+	11
Mannose	+	+	+	+	+	+	+
Fructose	13–30	20	42	13–33	30	32	9
Glycerol	75–178	ND	ND	61–205	130	84	270
Pyruvate	31–121§	9	30	5–22	48	25	25
Lactate	11–48	26	12	5–34¶	43	61	17
2-Oxobutyrate	32–65	+	ND	+	ND	18	21

* Except PP goat (rate 4).

† Except 607/79 (+).

‡ Except YP (ND), YO (ND).

§ Gladysdale strain 121, other strains < 76.

|| Gladysdale strain 48, other strains < 29.

¶ Except YP (153), YO (117).

Table 4. Rate of substrate utilization by *M. mycoides* subsp. *mycoides* LC strains

See Table 3 for substrate concentrations used and key to symbols.

Substrate	Initial rate of O ₂ uptake (percentage of initial rate for 25 μ M glucose)							
	Y-goat	400/79	81.636.1C	1645/82	221/82	F30	GE.6A.79E	Other strains (range)
Maltose	50	76	102	80	19	ND	ND	34–122
Trehalose	ND	ND	8	ND	ND	3	ND	ND*
GlcNAc	8	4	12	20	6	5	9	4–8†
Glucosamine	3	12	11	8	4	7	8	3–19
Mannose	11	52	75	+	+	23	34	23–58‡
Fructose	4	14	24	31	8	5	8	4–15
Glycerol	100	270	91	160	100	159	138	44–138
Pyruvate	15	43	36	16	6	14	12	5–30
Lactate	7	15	17	16	11	5	15	3–17
2-Oxobutyrate	7	32	46	ND	+	36	5	3–25

* Except 74/2488 (rate +), VRI (+), 977/79 (8) and 842/86 (8).

† Except OJO 1 (ND), KHI (ND), 78/441 (+), 11041 (+), 977/79 (+), 755/80 (+) and 842/86 (+).

‡ Except OJO 1 (3).

Table 5. Rate of substrate utilization by *M. mycoides* subsp. *capri* strains

See Table 3 for substrate concentrations used and key to symbols.

Substrate	Initial rate of O ₂ uptake (percentage of initial rate for 25 µM glucose)							
	NCTC 10137	BQT	Pendik	ZZ	JM	Isolates from Brazil*	G169/Leite	Other strains (range)
Maltose	35	47	42	35	43	49–71	ND	48–66
Trehalose	72	41	ND	ND	ND	6–10	5	ND†
GlcNAc	15	5	+	ND	ND	+	5	+
Glucosamine	35	7	9	13	4	7–14	6	7–11
Mannose	50	70	68	47	2	34–48	27	33–95
Fructose	18	28	14	12	5	5–14	10	10–15
Glycerol	230	250	106	77	88	76–126	67	80–220
Pyruvate	20	27	+	ND	10	5–17	26	19–35
Lactate	12	20	2	3	7	7–11	15	7–16
2-Oxobutyrate	+	78	+	5	16	4–15	31	30–40

* G105/A1, G108/A2a, G108/A2b, G108/A3.

† Except YC (rate +).

LC strains appeared metabolically diverse. Table 4 shows rates of substrate utilization for the reference strain (Y-goat), a number of strains with individually distinctive characteristics and the remaining more homogeneous strains. The reference strain oxidized all substrates tested except trehalose; it oxidized mannose and glucosamine at relatively low rates but appeared otherwise typical of the group. Strains 1645/82 and 221/82 had little or no activity towards mannose and 2-oxobutyrate, and two strains (F30 and GE.6A.79E) failed to oxidize maltose. Strains 81.63.61C and 400/79 oxidized the organic acids (lactate, pyruvate and 2-oxobutyrate) at particularly high rates; in contrast, for a number of strains (OJO 1, 78/441, 11041, VRI, COV2), the maximum rate of oxygen uptake in the presence of organic acids (50 µM) was < 10% of the rate with glucose.

The *M. mycoides* subsp. *capri* strains were also diverse (Table 5), although four of the five Brazilian strains gave very similar patterns. Strains differed particularly in their abilities to oxidize trehalose, GlcNAc and 2-oxobutyrate. In addition, two strains (Pendik and ZZ) oxidized organic acids at low rates and the Brazilian strain G169/Leite did not metabolize maltose although this substrate was metabolized by all other subsp. *capri* strains at high rates.

Bovine serogroup 7 strains. The Bovine serogroup 7 strains (Table 3) were distinguished from strains of the three *M. mycoides* groups (Tables 3–5) by their high rate of trehalose metabolism coupled with their inability to oxidize mannose and glucosamine at low concentrations (< 50 µM). However, they varied in their ability to metabolize GlcNAc and 2-oxobutyrate.

Subssp. *capricolum* and *capripneumoniae* strains. All subsp. *capricolum* strains metabolized maltose and GlcNAc

(Table 3). Mannose and (except for one strain) 2-oxobutyrate were also oxidized, but only in the presence of high substrate concentrations. Strains YP and YO were distinguished by their high rate of lactate (50 µM) oxidation, and strains California kid and 4161/88 by their inability to oxidize glycerol; glycerol was oxidized at high rates by all other strains.

The pattern of substrate oxidation by the subsp. *capripneumoniae* type strain (F38) was in marked contrast to that of *M. mycoides*, Bovine serogroup 7 and subsp. *capricolum* strains. Washed cell suspensions did not oxidize glucose, maltose or glycerol. However, pyruvate, lactate, 2-oxobutyrate and ethanol (10 mM) were rapidly metabolized (Table 6). A similar pattern of substrate oxidation to that of F38 was observed for strains G1943/80 and G94/83, though strain G183 oxidized glucose and glycerol and, at much lower rates, pyruvate and lactate. None of the other substrates tested were metabolized by strain G183, except maltose, which appeared to stimulate O₂ uptake minimally in some experiments (up to a value of 5% of the corresponding rate for glucose). However, this may reflect the relative difficulty in obtaining metabolically active cell suspensions of this organism which grew slowly and gave a relatively low cell yield. The type strain F38 also grew poorly in comparison with strains G1943/80 and G94/83.

Oxidation of ethanol (10 and 100 mM), observed in those subsp. *capripneumoniae* strains unable to oxidize glucose, was not detected in strain G183, nor in the subsp. *capricolum* strains listed in Table 1, nor in strains representing the other four groups of the *M. mycoides* cluster.

Patterns of substrate utilization amongst the six groups of the *M. mycoides* cluster are summarized in Table 7. Patterns

Table 6. Rate of substrate utilization by *M. capricolum* subsp. *capripneumoniae* strains

Substrate	Initial rate of O ₂ uptake (nmol min ⁻¹ per 10 ⁹ cells)			
	NCTC 10192	G1943/80	G94/83	G183
Glucose (25 µM)	ND	ND	ND	3.0
Glycerol (50 µM)	ND	ND	ND	3.3
Pyruvate (50 µM)	8.9	20	16	0.1
Lactate (50 µM)	8.0	17	7.4	0.5
2-Oxobutyrate (50 µM)	9.3	19	14	ND
Ethanol (10 mM)	2.9	15	2.7	ND

ND, Not detected at the concentrations stated or ten times these concentrations.

Table 7. Patterns of substrate utilization amongst the constituent groups of the '*M. mycoides* cluster'

The Table shows the number of strains able to use each substrate at low concentration (12–50 µM, see text) and, in parentheses, at high concentration (up to 500 µM), if this increased the number of positive strains.

Substrate	<i>M. mycoides</i> subsp. <i>mycoides</i>			<i>M. capricolum</i>			Bovine serogroup 7
	SC	LC	subsp. <i>capri</i>	subsp. <i>capricolum</i>	subsp. <i>capripneumoniae</i>		
	No. of strains tested...				G183	others	
	5	19	13	7	1	3	3
Glucose	5	19	13	7	1	0	3
Maltose	0	17	12	7	1	0	3
Trehalose	0	4 (6)	7 (8)	0	0	NT	3
GlcNAc	5	12 (17)	3 (11)	6 (7)	0	NT	2
Glucosamine	0 (5)	19	13	4 (5)	0	NT	1 (3)
Mannose	0 (5)	17 (19)	13	0 (7)	0	NT	0 (3)
Fructose	5	19	13	7	0	NT	3
Glycerol	5	19	13	5	1	0	3
Pyruvate	5	19	11 (12)	7	1	3	3
Lactate	5	19	13	7	1	3	3
2-Oxobutyrate	5	17 (18)	11 (13)	0 (6)	0	3	2

NT, Not tested.

varied within all groups, except the SC group of *M. mycoides* subsp. *mycoides*. However, in general, there were consistent differences in patterns between groups.

DISCUSSION

The strains of the *M. mycoides* cluster varied both in their ability to oxidize the test substrates and in the affinity with which particular substrates were utilized (Table 2). Similarly, in a microcalorimetric study (Miles *et al.*, 1985), the K_m values for glucose, glycerol, fructose and GlcNAc metabolism by *M. mycoides* subsp. *mycoides* strain T1 were < 5 µM, whereas the values for glucosamine and mannose were 130 µM and 1.3 mM respectively. Such differences in K_m values are important, as substrates with high K_m values may not be present in sufficient concentrations *in vivo* to be utilized at significant rates. Furthermore, where K_m values for substrates are high, mycoplasma strains

may not be specifically adapted to their utilization. Thus, it appears that there are no mannose- or glucosamine-specific transport components in *M. mycoides* subsp. *mycoides* strain T1, and the inability of glucose-negative transport mutants [probably lacking glucose specific Enzyme IIB of the phosphoenolpyruvate:phosphotransferase (PEP:PTS) uptake system; Lee *et al.*, 1986] to metabolize these substrates suggests that their entry into cells depends on glucose-specific component(s). In this study, all the glucose-oxidizing strains (Tables 3–5) in which mannose or glucosamine metabolism was not detectable at 25 µM did use these sugars when the concentration was increased. Thus, it may be generally the case within the *M. mycoides* cluster that the glucose-specific component of the PEP:PTS system enables transport of glucosamine and mannose provided they are present at high concentrations. However, in some strains, specific transport components for mannose and/or glucosamine

may additionally be present. For example, in the *M. mycoides* subsp. *capri* type strain, PG3, the K_m value for mannose utilization was 3 μ M and the rate of mannose oxidation was not reduced in glucose-negative transport mutants (R. R. Taylor & R. J. Miles, unpublished). In comparisons of strains, therefore, the presence or absence of metabolism may not be as significant as the ability to metabolize low concentrations of substrate.

The ability to use specific substrates would not appear to be lost during *in vitro* cultivation in their absence. For example, maltose was metabolized by nearly all *M. mycoides* subsp. *capri* and LC strains although this substrate is not found in the serum-based media used for subculturing mycoplasmas (Miles & Lee, 1983). Thus, since the catabolic activities and distribution of mycoplasmas are restricted, differences in patterns of substrate utilization within the *M. mycoides* cluster may reflect variations in tissue specificity and/or in host physiology. All glucose-oxidizing strains also oxidized fructose (25 μ M), although oxygen uptake rates varied between 4 and 42% of the rate for glucose. The ability to utilize GlcNAc (25 μ M) at high rates was particularly associated with SC strains (Tables 3–5) whereas oxidation of mannose (25 μ M) at high rates was characteristic of *M. mycoides* subsp. *capri* strains and the majority of the LC strains. The ability to use GlcNAc and mannose is of particular interest since, although they are major constituents of mammalian glycoconjugates, they normally occur in body fluids as free sugars only at very low concentrations. However, host-derived *N*-acetylglucosaminidase and mannoside activities (principally from lysosomes) may be released into cell surroundings following cellular damage.

The ability to utilize the disaccharides maltose and trehalose is also of interest. These sugars are rapidly hydrolysed in serum (Miles & Lee, 1983) and *in vivo* would not be available at sites accessible to serum enzymes. The ability of several *Mycoplasma* strains to oxidize trehalose also appears anomalous. This substrate does not appear to be present in mammalian tissues. It is possible that trehalose might be transported by a disaccharide uptake system, for which maltose is the principal substrate. However, a number of *Mycoplasma mycoides* subsp. *capri* strains (Table 5) oxidized trehalose (12 μ M) at high rates, suggesting the presence of a specific trehalose uptake system. Also, strain G169/Leite oxidized trehalose but not maltose.

All strains oxidized pyruvate (50 μ M) and/or lactate (50 μ M). These substrates apparently require a common component for transport in *M. mycoides* subsp. *mycoides* strain T1 (Lee *et al.*, 1986), and with the Gladysdale SC strain both substrates (50 μ M) stimulated high oxygen uptake rates (Table 3). In contrast, uptake rates with the Pendik and ZZ strains of subsp. *capri* were low (< 3% of the rate for glucose). The significance of these differences is unclear. Pyruvate may be used as an energy source for the growth of *M. mycoides* subsp. *capri* (Miles *et al.*, 1988) and the concentration of lactate and pyruvate in body fluids is sufficiently high (e.g. 0.5 and 0.05 mM respectively in bovine serum; Altman & Dittmer, 1974) to suggest that many strains are adapted to utilize these

substrates *in vivo*. The ability to oxidize lactate at high rates (Fig. 2b) has also been proposed as a pathogenicity factor in, for example, *Neisseria gonorrhoeae* (Britigan *et al.*, 1988), reducing the O_2 concentration and thus limiting the ability of neutrophils to generate reactive oxygen intermediates. 2-Oxobutyrate, an intermediate in threonine degradation (Rodwell, 1960), was oxidized at a high rate by all SC strains, whereas amongst the other groups this ability varied. The structural similarity between 2-oxobutyrate and pyruvate suggests that they might be substrates for the same uptake system. However, there was little correlation between the rates of pyruvate and 2-oxobutyrate oxidation in individual strains.

Nearly all strains oxidized glycerol at high rates. In *M. mycoides*, glycerol is metabolized via an L- α -glycerophosphate oxidase in a reaction which yields 1 mol H_2O_2 (mol glycerol)⁻¹. The quantity of H_2O_2 produced during glycerol oxidation is much greater than during oxidation of sugars or lactate (Miles *et al.*, 1991). This may be of significance *in vivo*, since H_2O_2 production is potentially important in mycoplasma pathogenicity (Tryon & Baseman, 1992). Glycerol oxidation might also be useful as a strain marker in epidemiological studies of subsp. *capri-colum*, in which group it was oxidized by only four out of six strains.

A principal reason for determining patterns of substrate utilization in the *M. mycoides* cluster is their potential application to the identification of new isolates, using either the simple and rapid d.o.t. procedure described here, or alternative methods. The type strains of a range of fermentative bovine, caprine and ovine mycoplasma species have previously been shown to utilize distinct combinations of substrates (Wadher *et al.*, 1990). However, in this study, biochemical diversity within the groups of the *M. mycoides* cluster was evident and some *M. mycoides* subsp. *capri* strains gave patterns of substrate utilization which were not readily distinguishable from those of *M. mycoides* subsp. *mycoides* LC strains. The similarity of PAGE protein patterns between LC and *capri* strains has previously been noted (Costas *et al.*, 1987; Leach *et al.*, 1989; Rodwell & Rodwell, 1978). However, in general, there were consistent differences (Table 7) in the patterns of substrate utilization between the various groups of the *M. mycoides* cluster. In particular, SC strains of *M. mycoides* were distinguished by their inability to oxidize maltose, trehalose and (at low concentrations) mannose and glucosamine. Such differences are of potential value in identification, though the observed biochemical diversity within groups may necessitate testing further strains, particularly Bovine serogroup 7 strains, of which only three were included in this study. It is also possible that, in some cases, the apparent biochemical diversity between isolates assigned to species or subgroups may mirror the difficulty in the serological typing of strains. For example, the Brazilian isolates of *M. mycoides* subsp. *capri* included strain G169/Leite which did not oxidize maltose and thus differed from all other *capri* strains. A serological difference has also been noted, however, in indirect immunofluorescence tests (Leach *et al.*, 1989), in which antiserum to *M. mycoides* subsp. *capri*

(strain PG3) had a low titre (10) for G169/Leite, but a high titre (100–400) for the other Brazilian strains. Similarly, antiserum prepared against the Gladysdale SC strain of *M. mycoides* subsp. *mycoides* had a high antibody titre (> 1000) against the two *M. mycoides* LC strains (F30 and GE.6A.79E) that, like all SC strains, failed to ferment maltose.

Surprisingly, the type strain F38 and two further subsp. *capripneumoniae* isolates (G1943/80 and G94/83) differed from all other strains in the study in that they did not oxidize glucose or other sugars. They did, however, oxidize pyruvate, lactate, 2-oxobutyrate and ethanol at high rates (Table 6). This pattern of substrate utilization is similar to that of *M. agalactiae*, *M. bovis genitalium*, *M. bovis* and *M. californicum* (Wadher *et al.*, 1990). The subsp. *capripneumoniae* strains also oxidized ethanol; the likely end-product of its metabolism is acetate, and since oxidation presumably occurs via NAD⁺ reduction with no energy conservation, the role of alcohol dehydrogenase activity is unclear.

Since the subsp. *capripneumoniae* strains do not oxidize sugars, they presumably lack both a PEP:PTS system and much of the Embden–Meyerhof pathway. The marked metabolic differences between these strains and subsp. *capricolum* strains is in contrast to the genetic and other evidence (Anon., 1991; Bonnet *et al.*, 1993) used in proposing the *M.* F38 group as a subspecies of *M. capricolum* (Leach *et al.*, 1993). However, Salih *et al.* (1983) have also shown, by electrophoretic analysis of a number of key enzyme activities, that F38-like strains differ considerably from those of subsp. *capricolum* and other members of the *M. mycoides* cluster. A single subsp. *capripneumoniae* isolate (G183) did oxidize glucose and glycerol but, unlike all subsp. *capricolum* strains, it did not oxidize GlcNAc and maltose was oxidized only at a low rate.

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REFERENCES

- Altman, P. L. & Dittmer, D. S. (1974). *Biology Data Book*, vol. 3, 2nd edn, p. 1963. Bethesda, MD: Federation of American Societies for Experimental Biology.
- Anon. (1979). International Committee on Systematic Bacteriology. Subcommittee on the Taxonomy of Mollicutes. Proposal of minimal standards for descriptions of new species of the class Mollicutes. *Int J Syst Bacteriol* **29**, 172–180.
- Anon. (1991). International Committee on Systematic Bacteriology. Subcommittee on the Taxonomy of Mollicutes, Minute 11. *Int J Syst Bacteriol* **41**, 333–336.
- Archer, D. B. (1979). Immunoprecipitation of Triton X-100-solubilized *Mycoplasma mycoides* proteins. *J Gen Microbiol* **115**, 111–116.
- Bonnet, F., Saillard, C., Bové, J. M., Leach, R. H., Rose, D. L., Cottew, G. S. & Tully, J. G. (1993). DNA relatedness between field

isolates of *Mycoplasma* F38 group, the agent of contagious caprine pleuropneumonia, and strains of *Mycoplasma capricolum*. *Int J Syst Bacteriol* **43**, 597–602.

Britigan, B. E., Klapper, D., Svendsen, T. & Cohen, M. S. (1988). Phagocyte-derived lactate stimulates oxygen consumption by *Neisseria gonorrhoeae*. *J Clin Invest* **81**, 318–324.

Costas, M., Leach, R. H. & Mitchelmore, D. L. (1987). Numerical analysis of PAGE protein patterns and the taxonomic relationships within the '*Mycoplasma mycoides* cluster'. *J Gen Microbiol* **133**, 3319–3329.

Cottew, G. S. (1979). Caprine–ovine mycoplasmas. In *The Mycoplasmas*, vol. 2, pp. 103–132. Edited by J. G. Tully & R. F. Whitcomb. London: Academic Press.

Cottew, G. S., Breard, A., Da Massa, A. J., Ernø, H., Leach, R. H., Lefevre, P. C., Rodwell, A. W. & Smith, G. R. (1987). Taxonomy of the *Mycoplasma mycoides* cluster. *Isr J Med Sci* **23**, 632–635.

Freundt, E. A. (1983). Culture media for classic mycoplasmas. *Methods Mycoplasmol* **1**, 127–135.

Freundt, E. A. & Razin, S. (1984). Genus 1. *Mycoplasma*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 742–770. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

ter Laak, E. A. (1992). Contagious bovine pleuropneumonia: a review. *Vet Q* **14**, 104–110.

Leach, R. H., Costas, M. & Mitchelmore, D. L. (1989). Relationship between *Mycoplasma mycoides* subsp. *mycoides* ('large-colony' strains) and *Mycoplasma mycoides* subsp. *capri*, as indicated by numerical analysis of one-dimensional SDS-PAGE protein patterns. *J Gen Microbiol* **135**, 2993–3000.

Leach, R. H., Ernø, H. & MacOwan, K. J. (1993). Proposal for designation of F38-type caprine mycoplasmas as *Mycoplasma capricolum* subsp. *capripneumoniae* subsp. nov. and consequent obligatory relegation of strains currently classified as *M. capricolum* (Tully, Barile, Edward, Theodore & Ernø, 1974) to *M. capricolum* subsp. *capricolum* subsp. nov. *Int J Syst Bacteriol* **43**, 603–605.

Lee, D. H., Miles, R. J. & Beezer, A. E. (1986). Isolation and microcalorimetric characterisation of glucose-negative and pyruvate-negative mutants of *Mycoplasma mycoides* subsp. *mycoides*. *FEMS Microbiol Lett* **34**, 283–286.

MacOwan, K. J. & Minette, J. E. (1977). The role of mycoplasma F38 in contagious caprine pleuropneumonia (CCPP) in Kenya. *Vet Rec* **101**, 380–381.

Miles, R. J. & Lee, D. H. (1983). Carbohydrate utilisation by *Mycoplasma mycoides* subsp. *mycoides* strain T1 in a medium without serum. *Microbios Lett* **24**, 129–133.

Miles, R. J. & Wadher, B. J. (1990). Kinetics and pattern of substrate utilisation by *Mycoplasma* spp. *Zentralbl Bakteriol Suppl* **20**, 675–677.

Miles, R. J., Beezer, A. E. & Lee, D. H. (1985). Kinetics of utilization of organic substrates by *Mycoplasma mycoides* subsp. *mycoides* in a salts solution: a flow-microcalorimetric study. *J Gen Microbiol* **131**, 1845–1852.

Miles, R. J., Wadher, B. J., Henderson, C. L. & Mohan, K. (1988). Increased yields of *Mycoplasma* spp. in the presence of pyruvate. *Lett Appl Microbiol* **7**, 149–151.

Miles, R. J., Taylor, R. R. & Varsani, H. (1991). Oxygen uptake and H₂O₂ production by fermentative *Mycoplasma* spp. *J Med Microbiol* **34**, 219–223.

Nascimento, E. R., Nascimento, M. Da G. F., Freundt, E. A. & Anderson, H. (1986). Isolation of *Mycoplasma mycoides* from outbreaks of caprine mycoplasmosis in Brazil. *Br Vet J* **142**, 246–257.

Okoh, A. E. J. & Ocholi, R. A. (1986). Disease associated with

Mycoplasma mycoides subspecies *mycoides* in sheep in Nigeria. *Vet Rec* **118**, 212.

Rodwell, A. W. (1960). Nutrition and metabolism of *Mycoplasma mycoides* var. *mycoides*. *Ann NY Acad Sci* **79**, 499–507.

Rodwell, A. W. & Mitchell, A. (1979). Nutrition, growth and reproduction. In *The Mycoplasmas*, vol. 1, pp. 103–139. Edited by M. F. Barile & S. Razin. New York: Academic Press.

Rodwell, A. W. & Rodwell, E. S. (1978). Relationships between strains of *Mycoplasma mycoides* subsp. *mycoides* and *capri* studied by two-dimensional gel electrophoresis of cell proteins. *J Gen Microbiol* **109**, 259–263.

Salih, M. M., Simonsen, V. & Ernø, H. (1983). Electrophoretic analysis of isoenzymes of *Mycoplasma* species. *Acta Vet Scand* **24**, 14–33.

Tryon, V. V. & Baseman, J. B. (1992). Pathogenic determinants and mechanisms. In *Mycoplasmas: Molecular Biology and Pathogenesis*, pp. 457–471. Edited by J. Maniloff, R. N. McElhaney, L. R. Finch & J. B. Baseman. Washington, DC: American Society for Microbiology.

Wadher, B. J. & Miles, R. J. (1988). α -Glucosidase in *Mycoplasma mycoides* subsp. *capri*. *FEMS Microbiol Lett* **49**, 459–462.

Wadher, B. J., Varsani, H., Abu-Groun, E. A. & Miles, R. J. (1990). Biochemical characterisation of bovine, caprine and ovine mycoplasmas. *Abstracts of the 8th Congress of the International Organisation for Mycoplasmaology (IOM)*, Istanbul, Turkey. *IOM Lett* **1**, 517–518.

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