



Short communication

Biochemical characterisation of some non fermenting, non arginine hydrolysing mycoplasmas of ruminants

Liaqat Ali Khan^a, Guido R. Loria^b, Ana Sofia Ramirez^c,
Robin A.J. Nicholas^{e,*}, Roger J. Miles^{a,1}, Mark D. Fielder^d

^a Department of Life Sciences, King's College London, 150 Stamford Street, London SE1 9NN, UK

^b Istituto Zooprofilattico Sperimentale della Sicilia, Palermo, Italy

^c Universidad de Las Palmas de Gran Canaria, Facultad de Veterinaria, Las Palmas, Spain

^d School of Life Sciences, Kingston University, Penrhyn Road, Kingston Upon Thames, KT1 2EE, UK

^e Veterinary Laboratories Agency (Weybridge) New Haw, Addlestone, Surrey KT 15 3NB, UK

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Abstract

The pattern and kinetics of substrate oxidation by type and recent field strains of *Mycoplasma agalactiae*, *Mycoplasma bovis*, *Mycoplasma bovigenitalium* and *Mycoplasma ovine/caprine* serogroup 11 were investigated by measurement of oxygen uptake. Metabolism of a range of organic acids, sugars and alcohols was detected. All the test strains were unable to oxidise sugars, glycerol and the organic acids, fumarate, malate and α -ketoglutarate (1 mM). All strains oxidised organic acid L-lactate, 2-oxobutyrate and pyruvate and demonstrated the ability to oxidise alcohols, particularly isopropanol, which was oxidised at a high rate and high affinity (0.5 mol/mol isopropanol). Its oxidation was consistent with acetone formation, which may be of important in relation to pathogenicity. All strains oxidised similar substrates, however differences were observed between strains in terms of the relative rates and kinetic values for some substrates.

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Abbreviations: NADH, nicotinamide adenine dinucleotide reduced form; DOT, dissolved oxygen tension; HEPESN-(2-hydroxyethyl) piperazine-*N'*-(ethansulphonic acid); ATPadenosine triphosphate

* Corresponding author. Tel.: +44 1932 357379; fax: +44 1932 357423.

E-mail address: r.a.j.nicholas@vla.defra.gov.uk (Robin A.J. Nicholas).

¹ Deceased.

1. Introduction

Mycoplasma agalactiae and *Mycoplasma bovis* are closely related pathogens and were originally classified within the same species. *M. agalactiae* is the principal cause of contagious agalactia in sheep and goats in Southern Europe and Asia. *M. bovis* is associated with mastitis, arthritis, pneumonia, genital and ocular disorders in cattle and is responsible for

25–33% of calf pneumonia in Britain whilst in the USA it may be involved in up to 50% of chronic calf pneumonias (Nicholas et al., 1999). *Mycoplasma ovine/caprine* serogroup 11 is known as a cause of infertility in sheep and was first isolated in the UK in 1998 (Nicholas et al., 1999). *Mycoplasma bovis*, first isolated in the UK from mastitic cattle (Davidson and Stuart, 1960), is biochemically very similar to *Mycoplasma* serogroup 11 (Da Massa et al., 1992). Clinically, *M. ovine* serogroup 11 and *M. bovis* also cause similar reproductive disorders, infertility and impaired sperm motility in their respective hosts (Ruhnke, 1994).

Biochemical characteristics are commonly used in diagnosis, often in conjunction with serological tests and PCR analysis to confirm the identity of the strain or species under examination. Mycoplasmas are dependent upon a wide range of precursors for macromolecule synthesis and energy generation, which is often derived from sugar fermentation, arginine hydrolysis, oxidation of certain organic acids or by a combination of these reactions (Miles, 1992). Therefore, the development of discriminating biochemical tests and metabolomic analysis will aid species identification, differentiation, and culture. Thus determination of other energy rich substrates utilised by different species and the rates and affinity with which they are used may be of significance in epidemiology and pathogenicity of these diseases and in the development of improved diagnostic assays.

Relatively little has been reported on the biochemistry of *M. agalactiae*, *M. bovis*, *M. bovis genitalium* and *M. ovine* serogroup 11. Therefore we have investigated the pattern, kinetics, distribution and specificity of organic acids and alcohols oxidising activities in *M. agalactiae*, *M. bovis*, *M. bovis genitalium* and *M. ovine* serogroup 11. The major aim was to identify distinctive metabolic features that might be useful in the identification of these species or by developing improved culture media.

2. Materials and methods

All strains were isolated from different disease outbreaks and were cloned by selection of single colonies from blood agar cultures. All strains were grown in broth medium, aliquoted and stored at

–70 °C. A full listing of the strains used including host species and geographical location are detailed in Table 1.

All mycoplasma strains were grown in a medium containing heat-inactivated porcine serum 100 ml/l, special peptone 20 g/l, yeast extract 5 g/l, glycerol 5 g/l, sodium chloride 5 g/l, HEPES 9 g/l, fresh yeast extract (Freundt, 1983) 100 ml/l and sodium pyruvate 5 g/l. The pH of the broth medium was 7.6.

Cell suspensions were obtained by dispensing 7 ml of broth medium in plastic screw capped tubes (Sterilin, UK) and inoculating with 0.1 ml of stored culture which had been thawed by warming at 37 °C for few minutes. Following the incubation of all strains at 37 °C (24 h), 0.1 ml quantities of these cultures were transferred to fresh medium and incubated similarly. The cells were harvested towards the end of exponential phase by centrifugation at $14,000 \times g$ for 4 min. Cultures of all strains were harvested after 20–24 h incubation, when the optical density (OD) at 540 nm had reached 0.20–0.25. The cells pellets were washed and resuspended in a Ringer-HEPES solution containing 18 g/l HEPES in one quarter strength Ringer's solution with catalase (C-10) 160 U/ml. The pH of the suspending medium was 7.6. The OD was adjusted to 1.00 at 540 nm, equivalent to 250–300 μg cell protein/ml and viable count 3×10^9 cfu/ml. Cell protein was determined by the method of Markwell et al. (1978). Viable counts were determined by plating 5 μl of cell suspensions, diluted in Ringer-HEPES on blood agar.

Substrate metabolism was determined from changes in dissolved oxygen tension (DOT) with an oxygen electrode system (Rank Brothers, Bottisham, Cambridge, UK) linked to a chart recorder and calibrated with air saturated water (Miles et al., 1998). Cell suspension (1 ml) in Ringer-HEPES solution plus catalase was added to the electrode vessel, which was magnetically stirred and maintained at 37 °C. The air saturated suspension was then isolated from air and test substrates were added by microsyringe. All test substrates were prepared at the appropriate concentrations on the day of use, using sterilised distilled water. Values for the saturation constants (K_s) were determined following the method of Miles et al. (1998). All experiments were repeated and data shown are mean values of three or more replicate independent experiments.

Table 1
Details of mycoplasma strains used in the current study

Species/strains	Host	Geographical origin
<i>M. bovis</i> NCTC 10131	Bovine	UK
79B96	Bovine	UK
81B96	Bovine	UK
82B96	Bovine	UK
119B96	Bovine	UK
193B99	Bovine	UK
67M98	Human	UK
135B99	Bovine	UK
136B99	Bovine	UK
137B99	Bovine	UK
139B99	Bovine	UK
142B99	Bovine	UK
156B99	Bovine	UK
5B00	Bovine	UK
10B00	Bovine	UK
12B00	Bovine	UK
<i>M. agalactiae</i> NCTC 10123	Ovine	UK
2193/91	Ovine	Italy
723/93	Ovine	Italy
499/93	Ovine	Italy
1070/93	Ovine	Italy
1209/93	Ovine	Italy
471/93	Ovine	Italy
453/94	Ovine	Italy
101/94	Ovine	Italy
730/97	Ovine	Italy
314/97	Ovine	Italy
<i>M. agalactiae</i> 423/98	Ovine	Italy
4400/99	Ovine	Italy
2245/99	Ovine	Italy
1536/99	Ovine	Italy
LF/00	Ovine	Italy
4 a	Ovine	Spain
10 a	Ovine	Spain
11 b	Ovine	Spain
6 gb	Ovine	Spain
A. Vet	Ovine	Spain
33328	Ovine	Spain
<i>M. bovigenitalium</i> NCTC 10122	Bovine	UK
398/87	Bovine	Germany
434/81	Bovine	Germany
57B00	Bovine	UK
Ovine serogroup 11		
Type strain 2D	Ovine	UK
48SR99	Ovine	UK
50SR99	Ovine	UK
52SR98	Ovine	UK
3SR99	Ovine	UK
47SR99	Ovine	UK
48SR99	Ovine	UK

Table 1 (Continued)

Species/strains	Host	Geographical origin
52SR99	Ovine	UK
95SR99	Ovine	UK
96SR99	Ovine	UK
126SR99	Ovine	UK
129SR99	Ovine	UK

3. Results

3.1. Organic acid oxidation

All *M. agalactiae* strains oxidised organic acids at relatively high rates. The K_s values were in the range of 5–12 μM for L-lactate (mean K_s values of $8.81 \mu\text{M} \pm 0.36$ S.E.M.), 7–23 μM for pyruvate (mean K_s values of $10.72 \mu\text{M} \pm 0.87$ S.E.M.) and 6–26 μM for 2-oxobutyrate (mean K_s values of $11.50 \mu\text{M} \pm 0.74$ S.E.M.) (See Fig. 1). Analysis using Tukeys multiple comparison tests indicated that the *M. agalactiae* strains showed significantly more affinity for lactate when compared to pyruvate whilst no significant difference was observed with 2-oxobutyrate. Strains of *M. bovis* showed a similar pattern of organic acid oxidation to those of *M. agalactiae*. In this data set the K_s values for the organic acids were also low: L-lactate 5–14 μM (mean K_s values of $9.06 \mu\text{M} \pm 0.65$ S.E.M.), pyruvate 8–13 μM (mean K_s values of $9.06 \mu\text{M} \pm 0.54$ S.E.M.) and for 2-oxobutyrate, 4–13 μM (mean K_s values of $10.93 \mu\text{M} \pm 0.40$ S.E.M.) (Fig. 1) showing overall the highest affinity for the substrates under examination. All 16 strains of *M. bovis* showed high relative rates (per cent of pyruvate) of oxygen uptake for organic acids a multiple comparison test (Tukeys) once again showed significant differences between the K_s values observed with different substrates. As was observed with data from *M. agalactiae*, the *M. bovis* strains pyruvate showed a significantly lower saturation constant when compared with either lactate or 2-oxobutyrate. All 16 *M. bovigenitalium* and *M. serogroup 11* strains also oxidised organic acids with high rates and low K_s values showing high affinity for the substrates. The K_s values were in the range of 9–13 μM for L-lactate, 8–12 μM for 2-oxobutyrate and 7–14 μM for pyruvate and 5–13 μM for L-lactate, 6–12 μM for 2-oxobutyrate and

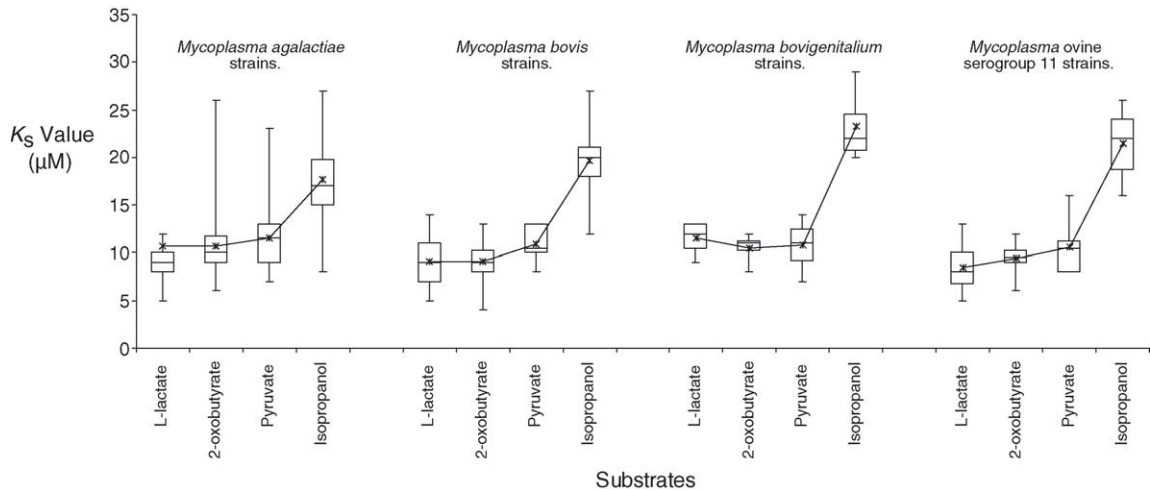


Fig. 1. Analysis of the K_s value (μM) data derived from the metabolism of different mycoplasma species against a range of substrates. Note: Boxes depict upper and lower quartiles of the data, the dark bar represents the median and error bars show minimum and maximum values with the joining line representing the mean K_s value.

8–16 μM for pyruvate, respectively. *M. bovigenitalium* showed a contrasting metabolomic pattern when compared to all other species under examination by showing a lower mean affinity for lactate when compared to 2-oxobutyrate or pyruvate, (Fig. 1; mean K_s values of 11.50, 10.50 and 10.70 $\mu\text{M} \pm 0.96$, 0.87, and 1.49 S.E.M., respectively). However, there is no statistical significance apparent in this trend. This lack of significance is repeated when examining the *M. ovine* serogroup 11 strains. Whilst this group shows the familiar metabolomic pattern with the greatest affinity shown to lactate > 2-oxobutyrate > pyruvate (mean K_s values of 8.40, 9.40 and 10.60 $\mu\text{M} \pm 0.72$, 0.48, and 0.73 S.E.M. respectively), using Tukey's multiple comparison test there is no significant difference between any of the strains.

3.2. Alcohol oxidation

Data obtained for alcohol oxidation showed great variation amongst strains tested as shown in Table 2 and clearly demonstrates the differences in K_s values achieved by the different type strains when oxidising the four alcohols under test. Clearly isopropanol showed the lowest K_s values across the range of strains tested showing a high affinity for the substrate. This observation contrasts with the other alcohols under test. For instance propanol was oxidised at very low

rates, and oxidation of this substrate was not detected at higher concentrations (10 mM). Ethanol and acetaldehyde (100 μM) were metabolised by some *M. agalactiae* strains at low rates, an observation reflected by the type strain which showed a K_s of only 103 and 270 μM , respectively (Table 2). Whilst acetaldehyde was oxidised by the type strain it was not

Table 2

Representative data of the kinetics of alcohol oxidation by the indicated *Mycoplasma* spp

Species ^a	Alcohol	K_s (μM)
<i>M. agalactiae</i> (NCTC 10123)	Isopropanol	15
	Propanol	>1 mM
	Ethanol	103
	Acetaldehyde	270
<i>M. bovis</i> (NCTC 10131)	Isopropanol	27
	Propanol	>1 mM
	Ethanol	Not detected
	Acetaldehyde	810
<i>M. bovigenitalium</i> (NCTC 10122)	Isopropanol	21
	Propanol	>1 mM
	Ethanol	50
	Acetaldehyde	>1 mM
<i>M. ovine</i> group 11 (Type strain 2D)	Isopropanol	18
	Propanol	500
	Ethanol	500
	Acetaldehyde	Not detected

^a Data are for type strains.

utilised by some field strains 314/97 and 423/98 (data not shown). Conversely, all *M. agalactiae* strains oxidised isopropanol at very high rates even when added at 25 μM concentration; these strains also showed low K_s values for isopropanol, 15–27 μM (data for type strain only, Table 2).

M. bovis NCTC 10131 type strain did not oxidise ethanol at high concentrations (1 mM) (Table 2) and low K_s values for isopropanol, 12–27 μM , were shown by all the *M. bovis* strains. The K_s values for propanol, ethanol, and acetaldehyde were high compared to isopropanol and showed lower affinity for these substrates as represented by the type strain (Table 2).

M. bovis genitalium and *M. ovine* serogroup 11 type strains also showed similar patterns of alcohol oxidation and the relative rates of isopropanol by all the strains were seen to be high (Table 2). Propanol, ethanol and acetaldehyde were also oxidised at lower velocity with relative rates of oxygen uptake and some strains (*M. bovis genitalium* strain 57B00) did not oxidise propanol, ethanol or acetaldehyde at higher concentrations (data not shown). The *M. serogroup* 11 type strain 2D oxidised ethanol at high K_s values (500 μM) along with a number of field strains demonstrating a low affinity for the substrate. Acetaldehyde metabolism was not detected by number of strains including the type strain 2D at high concentration (Table 2).

The values of K_s for isopropanol by *M. bovis genitalium* and *M. serogroup* 11 were also low 16–29 μM compared to other alcohols. The K_s values for propanol by all the strains were 360 μM –1 mM, for ethanol, 30 μM –1 mM. The K_s values for acetaldehyde were 205 μM –1 mM (data not shown). All strains of the four species have a similar pattern of alcohol oxidation with high affinity for isopropanol, with low affinity for other alcohols, with the exception of some strains which were unable to metabolise ethanol, propanol and acetaldehyde. Since K_s values for isopropanol were low, it was possible under the experimental conditions used to monitor its complete substrate oxidation by all these strains. Oxygen uptake following addition of isopropanol (100 μM) ceased quickly due to substrate exhaustion; further substrate addition yielded renewed oxygen uptake. The mean total oxygen uptake (five experiments) was 0.5 mol per mol. of isopropanol, the high affinity of isopropanol (low K_s values) and low affinity (high

K_s values) for other alcohols such as ethanol and propanol might be important as substrates with high K_s values may not be present in sufficient concentrations in vivo to be utilised at significant rates. Despite this high affinity for isopropanol amongst the organisms under test only *M. agalactiae* strains showed a greater affinity for the substrate when compared to the *M. ovine* serogroup 11 strains. There were no other statistical differences of significance between any other strain or group.

4. Discussion

Overall the substrate oxidation studies did not show any significant differences among *M. agalactiae*, *M. bovis*, *M. bovis genitalium* and *M. ovine* serogroup 11 and it can be clearly seen that there is only limited variation in mean organic acid oxidation between the strains tested. However, individual variances within the intra-strain group shows greater deviation from the mean with some substantial ranges of activity. This is most apparent with the *M. agalactiae* group with the least overall variation observed in the ovine serogroup 11 strains. It is tantalising to speculate that this variance in metabolic activity may have some consequence with regard to the pathology observed in clinical cases. However, this speculation requires further investigation. The other clear observation is the lower affinity of all strains for isopropanol. The data presented here show that the strains under examination here are adapted to a small range of the substrates and hence these four species are biochemically similar. The organic acids may serve as an energy source, this is not the case for isopropanol. The results obtained are potentially significant for improved culture media showing increased growth yields of these species as substrates have been identified for which the organisms have a high affinity. The observations also have potential in terms of pathology, as the utilisation of available substrates by invading mycoplasmas in vivo may, primarily, reduce bioavailability to host cells and, secondarily, result in the formation of toxic bi-products. Biochemically, distinctive metabolic features are potentially useful for strain identification, oxidation of isopropanol by *M. bovis* might be particularly useful as a confirmatory test and enable screening for this species when *Acholeplasma*

laidlawii (non-isopropanol oxidising species) is also present in the upper respiratory tract of cattle. Biochemical assays utilised in this study are important in establishing the presence of viable organisms rather than solely relying on the presence of nucleic acid which may not allow the discrimination between viable or non viable organisms. There is still a requirement for improved diagnostics in mycoplasmaology both in traditional culturing and in molecular/serological approach. Substrate utilisation assays such as those proposed in this study might be used where there are serological cross reactions. They may also help clarify epidemiological issues. There is a definite role for molecular epidemiology but biochemical variance amongst strains is also of potential value in the identification, containment and resolution of an outbreak. Such information may also have value in the distinction of certain species and sub specific groups. Currently, the only widely used biochemical tests are glucose fermentation and arginine hydrolysis and are not quantitative and routine detection of metabolism of other substrates during mollicutes growth is often not feasible because of low cells yields and difficulties in detecting the metabolism of specific substrates in a complex medium containing a wide range of alternative substrates. The data presented in this study along with the kinetic data shows the importance of continued assessment of substrate significance when

provided at physiological levels both in terms of culture media improvement and understanding the potential pathological importance of such metabolism.

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