SHORT COMMUNICATION

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SHORT COMMUNICATION

Kinetics of substrate oxidation and hydrogen peroxide production by *Mycoplasma mycoides* subsp. *mycoides* Large Colony (LC) type and *Mycoplasma mycoides* subsp. *capri*

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Abstract

Mycoplasma mycoides subsp. *mycoides* Large Colony (LC) type is a pathogen of goats causing contagious agalactia and respiratory disease, found on all continents where small ruminants are kept. It shares close genetic characteristics with *M. mycoides* subsp. *capri*. Substrate oxidation by 22 strains of *M. mycoides* subsp. *mycoides* LC from nine countries was compared with that of eight strains of *M. mycoides* subsp. *capri* from five countries. There was considerable similarity in the substrates used, but substrate saturation coefficients (K_s) varied for different substrates. Substrate utilization patterns and K_s values did not (1) significantly differentiate the LC strains from each other, (2) show any correlation with geographical origin, or (3) distinguish the LC strains from the *capri* strains. These results support previous studies justifying the reclassification of these subspecies as a single species.

Keywords: Mycoplasma mycoides; substrate oxidation; oxidation kinetics; taxonomy

In our characterization of mycoplasmas (Abu-Groun et al., 1994; Khan et al., 2005; Lin et al., 2008; Miles and Agbanyim, 1998; Shahram et al., 2008), we have analyzed the oxidation of ten substrates by strains of two very important pathogenic *Mycoplasma* subspecies. *M. mycoides* subsp. *mycoides* Large Colony (LC) type and *M. mycoides* subsp. *capri* can cause contagious agalactia, a serious disease of goats and sheep. LC strains have wide geographical distribution, occurring predominantly in goats on all habitable continents, wherever agalactia and caprine pleuropneumonia

are reported. *M. mycoides* subsp. *capri* shares genetic and immunological characteristics with the LC subspecies. Work by several groups, including our results from 16 LC and nine *capri* strains using PCR, restriction enzyme endonuclease analysis, protein profile patterns, growth inhibition, RAPD finger printing and 16S rRNA sequencing (M. Shahram, unpublished), indicates that all LC and *capri* strains should be classified as members of a single species: provisionally *"Mycoplasma capri"* or *"M. mycoides* subsp. *capri"* (Lin et al., 2008; Manso-Silvan et al., 2007; Minute 10, 2007; Monnerat et al., 1999; Pettersson et al., 1996; Vilei et al., 2006). We compared substrate oxidation and kinetics for 30 LC and *capri* strains from 13 countries (Table 1), to determine if the results (1) enabled differentiation among LC strains, (2) showed any correlation with the geographical origins of the strains, and (3) indicated identity of *Mycoplasma mycoides* subsp. *mycoides* LC and subsp. *capri* strains.

M. mycoides strains were grown at 37 ºC in a medium containing tryptose, yeast extract, glucose and HEPES buffer (Miles & Lee, 1983; Taylor et al., 1996), and harvested after growth for 12–16 hours, when OD_{550nm} was approximately 65 % of the maximum possible for each strain. Cultures harvested at maximum opacities were found to have greatly reduced metabolic activity. For substrate oxidation experiments, centrifuged organisms were washed twice with Ringer HEPES (RH) buffer (500 ml distilled water, 9g HEPES, one Oxoid Ringer tablet, adjusted to pH 7.6 with 10 M NaOH, supplemented with 800-2000 units catalase ml^{-1}), then resuspended in the same buffer. Catalase was included to prevent the reduction of activity and viability of the mycoplasmas, caused by H_2O_2 produced during substrate oxidation (Lin et al., 2008). Washing and resuspension of cells was completed within 15 min. Initially, cells were washed three times, but two washes were sufficient to remove residual

oxidizable substrates. The OD_{540nm} of cell suspensions was adjusted to 1.0, equivalent to 350 μg cell-protein ml^{-1}, and 10^9 colony-forming-units ml^{-1}.

Oxidation rates (Table 2) were determined polarographically (Clark-type oxygen electrode, model system 10; Rank Brothers, Cambridge, UK) in a cylindrical reaction vessel (diameter 15 mm, volume 5 ml), maintained at 37 °C. Initial dissolved oxygen tension (DOT, calibrated with distilled water) was 210 μM: changes in DOT were recorded using a Gould-BS 272 chart recorder. Cell suspensions (1 ml) were equilibrated at 37 °C for five minutes before injecting substrates with a Hamilton microsyringe. Initial oxidation rates were recorded and substrate saturation coefficients (K_s) calculated from Lineweaver-Burk plots of reciprocals of oxygen consumption rates against substrate concentrations (Abu-Groun et al., 1994; Miles and Agbanyim, 1998; Miles et al., 1985). All experiments were internally replicated, and the results presented are the average of two to four experiments.

Maximum substrate oxidation rates were obtained with organisms harvested after 12–16 h growth, with rates falling by 50% and 65% for organisms harvested after 24 h and 48 h, respectively. Organisms were used as soon as possible after harvesting and washing, as activity fell by 60–70% after 3 h storage at either 4 ºC or 22 ºC. All strains of both *M. mycoides* LC and *capri* subspecies oxidized glucose at comparable rates and with similar high affinity K_s values (Table 2): the rates of oxidation were identical with either 25 μM or 1 mM glucose. Oxygen consumption was about 2 mol O_2 (mol glucose)$^{-1}$, as expected for complete oxidation of glucose to equimolar acetate and CO_2. Rates ranged from about 33 nmol O_2 min^{-1} (mg cell-protein)$^{-1}$ for strain SP266, to 113 for strain GR50, but the ranges observed did not allow discrimination either within or between the two subspecies.

Relative to glucose, most other substrates were oxidized mainly at similar rates and affinities by each subspecies and within each group (Table 2). Oxidation rates and K_s values were broadly similar for all strains for the use of fructose, mannose, maltose, N-acetylglucosamine (NAG), pyruvate, lactate and glycerol: fructose was, however, not oxidized by LC strains SP266, CH5, CH6 or IT39se, and NAG was not used by *capri* strain JM. While failure of some strains to use some substrates may assist individual strain characterization, these differences did not discriminate between most strains of each subspecies or between the subspecies. There was some variation among strains in affinity for NAG, K_s ranging from 1.8–4.0 µM for the *capri* strains, and 0.4–2.0 µM for most LC strains: a few LC strains, including IT247, showed values around 12 µM. The affinities of all strains for glucosamine were poor (Table 2), ranging between 170–460 µM for the *capri* strains and 110–300 µM for most of the LC strains, with LC strains IT247, IT39se and PT994 showing K_s values of 2.0, 2.5 and 3.0 mM respectively. This might indicate a geographically-significant difference among LC strains, with those from Italy and Portugal having much lower affinities for glucosamine. For all strains, affinities for 2-oxobutyrate were lower overall than those for glucosamine (Table 2), ranging from 1.0–2.5 mM for *capri* strains and 1.0–2.1 mM for LC strains. Consequently, when tested at 400 µM (Table 2), oxidation rates were poor (at 8–20% of the glucose rates for the *capri* strains and 3–31% for the LC strains), as this concentration was considerably below the half-saturation concentration for all strains. While these results were metabolically interesting, they again did not enable distinction between strains or subspecies.

Production of H_2O_2 by all the strains was determined, as H_2O_2 is probably a contributory factor to the pathogenicity of mycoplasmas (Kannan and Baseman, 2000; Niang et al., 1998; Nicholas et al., 1996; Tryon and Baseman, 1992). Substrate

oxidation-dependent H_2O_2 production by lysed organisms was determined polarographically with cell suspensions (1ml), washed in catalase-free RH buffer, equilibrated at 37 ºC, and then lysed by injection of 10 µl Triton X-100. NADH (200 µM) or L-α-glycerophosphate (20 µM) were added, and decrease in DOT recorded to measure NADH oxidase and L-α-glycerophosphate oxidase. Catalase solution (10 µl, 40 mg ml^{-1}) was injected, and the amount of H_2O_2 formed calculated from the increase in DOT. H_2O_2 production showed that all strains possessed NADH oxidase, producing small amounts of H_2O_2: 0.05 mol (mol O_2)$^{-1}$ (LC strain GR51) to 0.13 mol (mol O_2)$^{-1}$ (LC strain CH5), showing similarity, rather than differences, among all the strains. This result was expected as NADH oxidase in most mycoplasmas produces only small amounts of H_2O_2 (<0.1 mol H_2O_2 per mol O_2 consumed; Miles et al., 1991; Taylor et al., 1996). All strains possessed L-α-glycerophosphate oxidase, producing 0.9 mol H_2O_2 (mol O_2)$^{-1}$ (LC strain GR52) to 1.25 mol H_2O_2 (mol O_2)$^{-1}$ (LC strain GR60), with a mean of 1 mol H_2O_2 (mol O_2)$^{-1}$. This indicated that glycerol metabolism involved L-α-glycerophosphate oxidase, which gives this stoichiometry (Miles et al., 1991), rather than NAD^+-dependent glycerol dehydrogenase coupled with NADH oxidase. Glycerol metabolism might thus produce significant amounts of H_2O_2 in the host, potentially enhancing the pathogenicity of the mycoplasmas.

These results significantly extend earlier observations on the biochemical capabilities of 30 *M. mycoides* subsp. *mycoides* LC and subsp. *capri* strains, with very few strains showing any differentiating characters on the basis of their geographical origin or subspecies-affiliation. This supports the amalgamation of the two subspecies into one, as inferred from other studies, including the >99.8% 16S rRNA gene sequence identity found among the strains we used (M. Shahram, unpublished).

References

Abu-Groun, E.A.M., Taylor, R.R., Varsani, H., Wadher, B.J., Leach, R.J. and Miles, R.J., 1994. Biochemical diversity in the '*M. mycoides*' cluster. *Microbiology*, 140, 2033-2042.

Kannan, T.R. and Baseman, J.B., 2000. Hemolytic and hemoxidative activities in *Mycoplasma penetrans*. *Infection and Immunity*, 68, 6419-6422.

Khan, L.A., Loria, G.R., Ramirez, A.S., Nicholas, R.A.J., Miles, R.J. and Fielder, M.D., 2005. Biochemical characterisation of some non-fermenting, non-arginine hydrolysing mycoplasmas of ruminants, *Veterinary Microbiology*, 109, 129-134.

Lin, Y-C., Agbanyim, C.N.M., Miles, R.J., Nicholas, R.A.J., Kelly, D.P. and Wood, A.P., 2008. Tetrazolium reduction methods for assessment of substrate oxidation and strain differentiation among mycoplasmas, including *Mycoplasma bovigenitalium* and *Mycoplasma mycoides* strains. *Journal of Applied Microbiology*, 105, 492-501.

Manso-Silvan, L., Perrier, X. and Thiaucourt, F. 2007. Phylogeny of the *Mycoplasma mycoides* cluster based on analysis of five conserved protein-coding sequences and possible implications for the taxonomy of the group. *International Journal of Systematic and Evolutionary Microbiology*, 57, 2247-2258.

Miles, R.J. and Lee, D.H. 1983. Carbohydrate utilization by *Mycoplasma mycoides* var *mycoides* strain T1 in a medium without serum. *Microbios Letters*. 24, 129-133.

Miles, R.J. and Agbanyim, C., 1998. Determination of substrate utilization rates by mycoplasmas. In: *Mycoplasma Protocols: Methods in Molecular Biology*, Miles, R.J. and Nicholas, R.A.J. (eds). Totowa, New Jersey: Humana Press, pp. 95-103.

181 Miles, R.J., Lee, D.H. and Beezer, A.E., 1985. Kinetics of utilization of organic
182 substrates by *Mycoplasma mycoides* subsp. *mycoides* in salt solution: a flow
183 microcalorimetric study. *Journal of General Microbiology*. 131, 1845-1852.
184 Miles, R.J., Taylor, R.R. and Varsani, H., 1991. Oxygen uptake and H_2O_2 production
185 by fermentative *Mycoplasma* spp. *Journal of Medical Microbiology*, 34, 219-223.
186 Minute 10. 2007. Minutes of the subcommittee on the taxonomy of Mollicutes (July
187 2002). *International Journal of Systematic and Evolutionary Microbiology*, 57,
188 183.
189 Monnerat, M-P., Thiaucourt, F., Poveda, J.B., Nicolet, J. and Frey, J., 1999. Genetic
190 and serological analysis of lipoprotein LppA in *Mycoplasma mycoides* subsp.
191 *mycoides* LC and *Mycoplasma mycoides* subsp. *capri. Clinical and Diagnostic*
192 *Laboratory Immunology*, 6, 224-230.
193 Niang, M., Rosenbusch, R.F., DeBey, M.C., Niyo, Y., Andrews, J.J. and Kaeberle, M.
194 L., 1998. Field isolates of *Mycoplasma ovipneumoniae* exhibit distinct cytopathic
195 effects in ovine tracheal organ cultures. *Journal of Veterinary Medicine*, 45, 29-
196 40.
197 Nicholas, R.A.J., Santini, F.G., Clark, K.M., Palmer, N.M.A., De Santis, P. and
198 Bashiruddin, J.B., (1996). A comparison of serological tests and gross lung
199 pathology for detecting contagious bovine pleuropneumonia in two groups of
200 Italian cattle. *Veterinary Record*, 139, 89-93.
201 Pettersson, B., Leitner, T., Ronaghi, M., Bölske, G., Uhlén, M. and Johansson, K.-E.
202 1996. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence
203 analysis of the 16S rRNA genes from the two rRNA operons. *Journal of*
204 *Bacteriology*, 178, 4131-4142.

205 Shahram, M., Nicholas, R.A.J., Miles, R.J., Kelly, D.P. and Wood, A.P., 2008. Sugar
206 uptake by *Mycoplasma mycoides* strain PT994 and *Mycoplasma canis* strain 85
207 B97. *Annals of Microbiology*, 58, 13-16.
208 Taylor, R.R., Mohan, K. and Miles, R.J., 1996. Diversity of energy-yielding
209 substrates and metabolism in avian mycoplasmas. *Veterinary Microbiology*, 51,
210 299-304.
211 Tryon, V.V. and Baseman, J.B., 1992. Pathogenic determinants and mechanisms. In
212 *Mycoplasmas: Molecular Biology and Pathogenesis*. Maniloff, J., McElhaney,
213 R.N., Finch L.R. and Basema J.B. (eds). Washington DC: American Society for
214 Microbiology, pp. 457-471.
215 Vilei, E.M., Korczak, B.M. and Frey, J. (2006) *Mycoplasma mycoides* subsp. *capri*
216 and *Mycoplasma mycoides* subsp. *mycoides* LC can be grouped into a single
217 species. *Veterinary Research*, 37, 779-790.

Table 1

Strains, and their sources, of *Mycoplasma mycoides* subsp. *mycoides* LC, and *M. mycoides* subsp. *capri* used in this study

Mycoplasma strains	Country of origin (and source[a])	Host /disease/site (where known)
***Mycoplasma mycoides* subsp. *mycoides* LC**		
Y-goat (NCTC 11706), 1141, 1164	Australia (1)	Goat/-/-
CH5, CH6	Chile (VLA[b])	Goat/respiratory/lung
FR755, FR1645	France (2)	Goat/-/milk
SP80, SP266	Spain (VLA)	Goat/respiratory/lung
SP152	Spain (VLA)	Goat/respiratory/pleural fluid
IT39se	Italy (3)	Goat/respiratory/milk
IT247	Italy (3)	Cattle/none/nasal swab
NZ67	New Zealand (VLA)	Goat/arthritis/joint fluid
NZ68	New Zealand (VLA)	Goat/respiratory/milk
PT994	Portugal (4)	Goat/respiratory/milk
GR50, GR60	Greece (VLA)	Goat/respiratory/pleural fluid
GR51, GR59	Greece (VLA)	Goat/respiratory/lung
GR52	Greece (VLA)	Calf/arthritis/joint fluid

GR55	Greece (VLA)	Kid/respiratory/lungs
GM12	USA (VLA)	
Mycoplasma mycoides* subsp. *capri		
JM	Australia (1)	Goat/-/-
Pendik, BQT, PG3 (NCTC 10137)	Turkey (1)	Goat/-/-
N108	Nigeria (1)	Goat/-/-
G108	Kenya (1)	Goat/-/-
G105 A1, G169	Brazil (1)	Goat/-/-

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[b] VLA – Strains from the collection of the Veterinary Laboratories Agency

Table 2

Relative oxidation rates and substrate saturation coefficients (K_s) of ten substrates for 22 strains of *Mycoplasma mycoides* subsp. *mycoides* LC and eight strains of *M. mycoides* subsp. *capri*.

Substrate (μM)	Relative oxidation rates (%)		K_s (μM)	
	LC strains	*capri* strains	LC strains	*capri* strains
Glucose[a] (25)	100	100	3.5 ± 1.7	3.5 ± 0.7
Fructose[b] (25)	21 ± 11	17 ± 6	2.5 ± 2.3	2.2 ± 0.9
Mannose (25)	51 ± 16	52 ± 12	2.6 ± 1.4	4.6 ± 2.2
Maltose (12)	72 ± 24	59 ± 7	1.8 ± 1.5	1.0 ± 0.5
Glucosamine[c] (25)	16 ± 11	15 ± 8	173 ± 51	271 ± 118
NAG[d] (25)	22 ± 7	11 ± 6	2.6 ± 4.0[e]	3.0 ± 0.9
Glycerol (50)	163 ± 29	181 ± 27	3.7 ± 2.0	3.5 ± 0.4
Pyruvate (50)	55 ± 27	33 ± 9	3.6 ± 1.2	3.7 ± 1.0
Lactate (50)	111 ± 41	75 ± 9	3.5 ± 1.7	3.5 ± 1.6
2-oxobutyrate[f] (400)	17 ± 10	14 ± 5	1380 ± 460	1710 ± 580

Means ± standard deviations from two to four experiments are shown.

[a] Mean O_2 uptake rates for all strains were 73 ± 20 nmol min^{-1} (mg cell-protein) $^{-1}$.

[b] Fructose was not oxidized by LC strains SP266, CH5, CH6 or IT39se.

[c] K_s values for glucosamine for strains IT247, PT994 and IT39se were 2–3 mM.

[d] NAG = N-acetylglucosamine. NAG was not oxidized by *capri* strain JM.

[e] The K_s for most strains, 0.8 ± 0.6 μM, but some, including IT247, were up to 12 μM.

[f] 2-oxobutyrate was not oxidized by LC strains SP266, CH5 or CH6.