Isoenzymes in Two Species of Acholeplasma

By S. M. Lanham,1* R. M. Lemcke,2† C. M. Scott1
AND J. M. Grendon1

1Department of Medical Protozoology, London School of Hygiene and Tropical Medicine, London WC1E 7HT
2Lister Institute, London SW1W 8RH

(Received 30 May 1979; revised 17 August 1979)

The reference strains Type A and Type B and two equine strains of Acholeplasma laidlawii were examined for a wide range of isoenzymes using thin-layer starch-gel electrophoresis; in addition two isoenzymes were examined in two strains of A. equifetale. The type strains A and B of A. laidlawii were differentiated by their lactate dehydrogenase, phosphoglucomutase and aspartate aminotransferase patterns and the two equine strains by their hexokinase, lactate dehydrogenase and phosphoglycerate kinase patterns. The two pairs of strains differed from one another with respect to hexokinase, phosphoglucomutase, adenylate kinase and glucose-6-phosphate dehydrogenase. The two strains of A. equifetale could be distinguished by their isoenzymes of hexokinase. The two species were differentiated by their hexokinase and phosphoglucomutase patterns.

INTRODUCTION

Mycoplasmas belonging to the genera Acholeplasma and Mycoplasma are grouped into species, mainly on a serological basis, by a variety of tests (Freundt et al., 1979). Some of these methods, for example, agglutination, indirect haemagglutination and metabolic inhibition, indicate the existence of antigenic heterogeneity among the strains of certain species (Nicol & Edward, 1953; Taylor-Robinson et al., 1965; Purcell et al., 1967). Intraspecific protein differences have also been demonstrated by immunodiffusion (Lemcke, 1965; Hollingdale & Lemcke, 1970), polyacrylamide gel electrophoresis (Razin, 1968; Forshaw, 1972) and two-dimensional immunoelectrophoresis (Thirkill & Kenny, 1974). In species where DNA-RNA hybridization has been carried out, the existence of intraspecific heterogeneity has been confirmed (Reich et al., 1966a, b; Somerson et al., 1966). Nevertheless, it is still not clear which criteria should be used to establish subspecies. In consequence, only one species, M. mycoides, has so far been classified at an infraspecific level.

There is need to explore other properties which might provide suitable markers for characterizing mycoplasmas at the infraspecific level. Such markers might be found amongst the isoenzymes of mycoplasmas since differences in enzyme structure when associated with a dissimilarity in net electric charge can be demonstrated by electrophoresis and have been used to study the taxonomic relationships of many organisms ranging from man to bacteria (Harris & Hopkinson, 1976; Markert, 1975). In the parasitic protozoa, isoenzyme electrophoresis has proved of value in species and subspecies differentiation, as for example, within the genera Entamoeba (Sargeaunt & Williams, 1978), Leishmania (Al-Taqi & Evans, 1978), Plasmodium (Carter, 1973) and Trypanosoma (Godfrey & Kilgour, 1976; Miles et al., 1977). Applications of the technique to the bacteria have included the corynebacteria (Robinson, 1966), Enterobacteriaceae (Bowman et al., 1967; Baptist et al., 1969), lactobacilli

† Present address: Agricultural Research Council Institute for Research on Animal Diseases, Compton, Newbury, Berkshire RG16 0NN.
mycoplasmas not requiring sterol (achopleasmas) are more easily lysed than the sterol-
requiring mycoplasmas, and enzymes are less likely to be inactivated during the preparation
of lysates. Moreover, A. laidlawii seemed a particularly suitable subject in which to explore
enzymic diversity because of conflicting evidence about the extent of variation within the
species. Laidlaw & Elford (1936) originally distinguished Type A from Type B on the basis
of agglutination tests, but several other serological tests as well as electrophoresis of cell
proteins failed to distinguish the two types (Lemcke, 1964; Tully, 1973; Hinz & Neumann,
1978). DNA–RNA hybridization showed only a 70% homology between Types A and B, a
degree of homology similar to that found between strains recognized as distinct species in
the genus Pseudomonas (McGee et al., 1967). Apart from the observations of Tully (1973)
on differences in mannose fermentation and aesculin hydrolysis, little comparative work has
been done on the metabolic activities of different strains of A. laidlawii.

METHODS

Organisms. Acholeplasma laidlawii strains PG8 (Type A) and PG9 (Type B) were National Institutes of
Health Research Reagents. These constitute reference strains since they were the first isolates of the species,
originating from sewage (Laidlaw & Elford, 1936); strain PG8 is the type strain (Edward & Freundt, 1973).
Strains 358 and N99 were isolated from the respiratory tracts of horses and identified as A. laidlawii by growth
and metabolic inhibition tests (Allam & Lemcke, 1975). Acholeplasma equifetale strain C112 was isolated
from an aborted equine foetus and has been designated as the type strain of the species (Kirchhoff, 1978).
Acholeplasma equifetale strain N93 was isolated from the respiratory tract of a horse and identified sero-
logically with the type strain (Allam & Lemcke, 1975). All the strains included in the study had been cloned
by filtration and plating, strains PG8 and PG9 before inclusion in the Research Reagent programme and the
others in the laboratories in which they were isolated.

Medium and preparation of samples. Acholeplasma laidlawii strains PG8, PG9, 358 and N99 were grown
in 4 l batches of Difco PPLO broth supplemented with a 25% aqueous extract of dried yeast (10%, v/v),
Difco PPLO serum fraction (1%, v/v), sodium deoxyribonucleate (0-002%, w/v), glucose (0.5%, w/v),
phenol red (0-002%, w/v) and ampicillin (300 μg ml⁻¹) and adjusted to pH 7.8. When the pH had fallen
to between 7.2 and 7.3 (22 to 25 h), organisms were harvested by centrifugation at 8000 g for 30 min and washed
twice in 0.25 M-NaCl buffered to pH 7.0 with Na₂HPO₄. Washed organisms from a 4 l culture were resus-
pended in 12 ml of an enzyme stabilizing solution containing dithiothreitol, s-aminocaproic acid and EDTA
(each at 1 mm) at pH 7.0 (Kilgour & Godfrey, 1973). To lyse the organisms, suspensions were frozen using
dry ice/acetone and thawed by gentle agitation in lukewarm water. The lysate was centrifuged at 10000 g for
2 h at 4 °C to deposit the membranes. The supernatant was removed and stored in liquid nitrogen either
as 500 μl samples or as frozen beads of 10 to 20 μl, to await enzyme analysis. Samples were prepared by the
same method from 1-6 l cultures of A. laidlawii strains PG8 and PG9 and A. equifetale strains C112 and
N93 grown in medium from which the glucose was omitted. Portions of the stored supernatants of strains
PG8, PG9 and N99 were later thawed and concentrated in a Minicon-A25 multipurpose microconcentrator
(Amicon, High Wycombe, Bucks.). The concentrated samples were used for electrophoresis where prelimi-
nary experiments indicated low activity for a particular enzyme in the original extract.

Electrophoretic technique. The general procedure and apparatus used for thin-layer starch-gel electrophoresis of enzymes was based on the method of Wraxall & Culliford (1968) as modified by C. W. Parr
(personal communication) and more recently described and further modified by Kilgour & Godfrey (1973),
Bagster & Parr (1973), Kilgour et al. (1974) and Miles et al. (1977). The two different types of enzyme
development were well-known procedures based on the oxidation/reduction reaction of NAD(P)⁺-dependent
dehydrogenases [see Harris & Hopkinson (1976) for a comprehensive description of such methods]. Some
<table>
<thead>
<tr>
<th>Enzyme (EC no.)</th>
<th>Electrode buffer</th>
<th>Dilution for gel buffer</th>
<th>Voltage (cm⁻¹)</th>
<th>Time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate kinase (2.7.4.3)</td>
<td>0.05 M-Na₂HPO₄/0.007 M-citric acid pH 7.0</td>
<td>2:3</td>
<td>26</td>
<td>210</td>
<td>Carter (1973)</td>
</tr>
<tr>
<td>Alanine aminotransferase (2.6.1.2)</td>
<td>0.15 M-Tris/0.0075 M-citric acid pH 9.0</td>
<td>1:9</td>
<td>23</td>
<td>45</td>
<td>Kilgour &amp; Godfrey (1973)</td>
</tr>
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<td>Aspartate aminotransferase (2.6.1.1)</td>
<td>0.15 M-Glycine/NaOH pH 9.5</td>
<td>1:9</td>
<td>24</td>
<td>90</td>
<td>Godfrey &amp; Kilgour (1976)</td>
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<tr>
<td>Enolase (4.2.1.11)</td>
<td>0.18 M-Tris/0.03 M-KH₂PO₄ pH 9.0</td>
<td>1:3</td>
<td>18</td>
<td>155</td>
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<td>Glucose-6-phosphate dehydrogenase (1.1.1.49)</td>
<td>0.15 M-Tris/0.1 M-KH₂PO₄ pH 8.3</td>
<td>1:9</td>
<td>19</td>
<td>120</td>
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</tr>
<tr>
<td>Glucosephosphate isomerase (5.3.1.9)</td>
<td>0.1 M-Tris/0.1 M-maleic acid/0.01 M-EDTA/0.01 M-MgCl₂* pH 7.4</td>
<td>1:9</td>
<td>15</td>
<td>165</td>
<td>Spencer et al. (1964)</td>
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<td>Hexokinase (2.7.1.1)</td>
<td>0.029 M-LiOH/0.192 M-boric acid pH 8.1</td>
<td>1:9†</td>
<td>23</td>
<td>150</td>
<td>Scandalios (1969)</td>
</tr>
<tr>
<td>Lactate dehydrogenase (1.1.1.27)</td>
<td>0.15 M-Tris/0.1 M-KH₂PO₄ pH 8.3</td>
<td>1:9‡</td>
<td>21</td>
<td>150</td>
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<tr>
<td>Phosphoglucomutase (2.7.5.1)</td>
<td>0.1 M-Tris/0.1 M-maleic acid/0.01 M-EDTA/0.01 M-MgCl₂* pH 7.4</td>
<td>1:9</td>
<td>18</td>
<td>270</td>
<td>Spencer et al. (1964)</td>
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<tr>
<td>Phosphoglycerate kinase (2.7.2.3)</td>
<td>0.133 M-Tris/0.0317 M-citric acid/0.0066 M-EDTA pH 7.8</td>
<td>3:17</td>
<td>19</td>
<td>135</td>
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<td>Pyruvate kinase (2.7.1.40)</td>
<td>0.2 M-KH₂PO₄/KOH pH 7.0</td>
<td>3:37</td>
<td>15</td>
<td>180</td>
<td></td>
</tr>
</tbody>
</table>

* Tris, maleic acid and EDTA were neutralized at double strength with 10 M-NaOH, then MgCl₂ was added and the pH was adjusted to 7.4 with 10 M-NaOH at just under the final volume before completion.
† 1 vol. electrode buffer plus 9 vol. 0.2 M-Tris/0.0076 M-citric acid.
‡ Fructose 1,6-bisphosphate added to a final concentration of 1 mM.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Buffer</th>
<th>Coenzyme (mg ml⁻¹)</th>
<th>Substrate(s) (mg ml⁻¹)</th>
<th>Linking enzyme(s) (units ml⁻¹)</th>
<th>Activator(s)† (mm)</th>
<th>Dye† (mg ml⁻¹)</th>
<th>Reference</th>
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<tr>
<td>Adenylate kinase</td>
<td>0.1 M-Tris/HCl pH 8.0</td>
<td>NADP⁺ 0.40</td>
<td>ADP 0.95; glucose 1.80</td>
<td>Hexokinase 0.25; glucose-6-phosphate dehydrogenase 0.25</td>
<td>MgCl₂ 10.0; KCl 60.0; F1,6BP⁺ 5.0</td>
<td>MTT 0.25; PMS 0.05</td>
<td>Fildes &amp; Harris (1966)</td>
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<td>Alanine aminotransferase</td>
<td>0.1 M-Na₂HPO₄/NaH₂PO₄ pH 7.4</td>
<td>NADH 0.50</td>
<td>2-Oxoglutarate 1.67; L-alanine 20.80</td>
<td>Lactate dehydrogenase 15.0</td>
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<td>Godfrey &amp; Kilgour (1976)</td>
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<td>Aspartate aminotransferase</td>
<td>0.1 M-Na₂HPO₄/NaH₂PO₄ pH 7.4</td>
<td>NADH 0.50</td>
<td>2-Oxoglutarate 1.67; L-aspartic acid 4.20</td>
<td>Malate dehydrogenase 15.0</td>
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<td>Enolase</td>
<td>0.1 M-Tris/HCl pH 8.0</td>
<td>NADH 0.25</td>
<td>3-Phosphoglycerate 1.00; ADP 1.00</td>
<td>Phosphoglycerate mutase 2.5; pyruvate kinase 1.25; lactate dehydrogenase 2.5</td>
<td>MgCl₂ 5.0</td>
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<td>Harris &amp; Hopkinson (1976)</td>
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<td>Glucose-6-phosphate</td>
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<td>NADP⁺ 0.30</td>
<td>Glucose 6-phosphate 0.25</td>
<td>Glucose-6-phosphate dehydrogenase 0.25</td>
<td>MgCl₂ 10.0</td>
<td>MTT 0.25; PMS 0.05</td>
<td>Carter &amp; Parr (1967)</td>
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<td>dehydrogenase</td>
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<td>Glucosephosphate isomerase</td>
<td>0.1 M-Tris/HCl pH 8.0</td>
<td>NADP⁺ 0.25</td>
<td>Fructose 6-phosphate 0.25</td>
<td>Glucose-6-phosphate dehydrogenase 0.25</td>
<td>MgCl₂ 10.0</td>
<td>MTT 0.25; PMS 0.05</td>
<td>Carter &amp; Parr (1967)</td>
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<td>Hexokinase</td>
<td>0.1 M-Tris/HCl pH 7.4</td>
<td>NAD⁺ 1.60</td>
<td>Glucose 18.00; ATP 3.00</td>
<td>Glucose-6-phosphate dehydrogenase 0.5</td>
<td>MgCl₂ 5.0; KCN₂⁺ 2.0</td>
<td>MTT 0.25; PMS 0.05</td>
<td>Katzen &amp; Schimke (1965)</td>
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<td>Lactate dehydrogenase</td>
<td>(a) 0.05 M-Tris/</td>
<td>NADH 0.20</td>
<td>Pyruvate 0.55</td>
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<td>F1,6BP 1.0</td>
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<td>(b) 0.05 M-Tris/</td>
<td>NAD⁺ 0.25</td>
<td>Lactate 0.78</td>
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<td>F1,6BP 1.0</td>
<td>MTT 0.125; PMS 0.062</td>
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<td>HCl pH 8.0</td>
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<td>Phosphoglucomutase</td>
<td>0.1 M-Tris/HCl pH 8.0</td>
<td>NAD⁺ 0.25</td>
<td>Glucose 1-phosphate (containing glucose-1,6-bisphosphate) 0.62</td>
<td>Glucose-6-phosphate dehydrogenase 0.25</td>
<td>MgCl₂ 10.0</td>
<td>MTT 0.25; PMS 0.05</td>
<td>Spencer et al. (1964)</td>
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<tr>
<td>Phosphoglycerate kinase</td>
<td>0.1 M-Tris/HCl pH 8.0</td>
<td>NADH 0.25</td>
<td>3-Phosphoglycerate 2.00; ATP 3.75</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase 5.0; triosephosphate isomerase 10.0; glyceraldehyde-3-phosphate dehydrogenase 10.0</td>
<td>MgCl₂ 12.5</td>
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<td>Scopes (1968)</td>
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<tr>
<td>Pyruvate kinase</td>
<td>(a) 0.05 M-Tris/</td>
<td>NADH 0.13</td>
<td>Phosphoenolpyruvate 0.55; ADP 1.00</td>
<td>Lactate dehydrogenase 2.0</td>
<td>MgCl₂ 20.0; KCl 75.0</td>
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<tr>
<td></td>
<td>(b) 0.05 M-Tris/</td>
<td>NAD⁺ 0.35</td>
<td>Phosphoenolpyruvate 0.40; ADP 0.55; glucose 1.80</td>
<td>Glucose-6-phosphate dehydrogenase 0.25; hexokinase 0.25</td>
<td>MgCl₂ 10.0; KCl 60.0; F1,6BP 5.0</td>
<td>MTT 0.25; PMS 0.05</td>
<td>Brewer (1970)</td>
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<td>HCl pH 7.4</td>
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</table>

* The developers for the aminotransferases were applied in a filter paper overlay, the remainder in an agar (6 mg ml⁻¹) overlay.
† F1,6BP, Fructose 1,6-bisphosphate; MTT, MTT tetrazolium salt; PMS, phenazine methosulphate.
‡ Optional.
of the conditions used for the electrophoresis and identification of the enzymes were our own, others were established methods or modifications thereof. The conditions for electrophoresis are given in Table 1 and the composition of developers for demonstrating enzyme activities in Table 2. Malate dehydrogenase (EC 1.1.1.37), ‘malic enzyme’ (EC 1.1.1.40), isocitrate dehydrogenase (NADP+) (EC 1.1.1.42) and phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) were also investigated, using the procedures given by Harris & Hopkinson (1976). Eight samples were applied to each starch gel (21.5 × 14.0 × 0.1 cm thick) by means of cotton threads, 1 cm long, placed in slots at a distance of 7 cm from the cathode (Kilgour & Godfrey, 1973; Kilgour et al., 1974). The temperature of the cooling plate was maintained at 8 °C during electrophoresis. Developer solutions were applied as agar overlays approximately 2 mm thick (final agar concentration, 6 mg ml⁻¹) except for the aminotransferase reagents which were applied on filter paper (Whatman no. 1) overlays. The overlaid plates were incubated at 37 °C in the dark until bands of a density suitable for photography were obtained. The incubation time varied from 15 to 180 min, depending on the nature and activity of the enzyme. For each enzyme examined, control plates were run on all extracts under the same electrophoretic and developmental conditions except that the main substrate was omitted. With the more complex developing reagents, additional controls were run with omission of other ingredients, such as co-substrates, activators or coupling enzymes. Samples were applied at comparable enzyme activities, being diluted when necessary in 5% sterile bovine serum albumin. Enzymes could usually be detected if used within an activity range from 0.2 to 1.0 μmol substrate transformed min⁻¹ ml⁻¹, depending on the enzyme and staining method. Where activities were very low, membrane-concentrated samples were sometimes used. Alternatively, additional material was loaded on to the cotton threads by repeated dipping and drying (V. Kilgour, personal communication): the thread was dipped in a sample, then dried on Parafilm under gentle heat from a bench lamp, this process being repeated 3 to 5 times before applying the thread to the gel. Samples were run at least twice on different occasions to ensure that results were reproducible; those with similar mobilities were also run as mixtures as a check on electrophoretic identity.

As is the case with all enzyme preparations, the conditions for the isolation and purification of the enzymes may affect the results. Some enzymes which are difficult to demonstrate in vivo may be shown in vitro. A number of isoenzymes have been investigated and the results of these studies are given in Table 2. The conditions for the electrophoresis and identification of the enzymes were established by others, or modifications thereof. The conditions for electrophoresis are given in Table 1 and the composition of developers for demonstrating enzyme activities in Table 2. Malate dehydrogenase (EC 1.1.1.37), ‘malic enzyme’ (EC 1.1.1.40), isocitrate dehydrogenase (NADP+) (EC 1.1.1.42) and phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) were also investigated, using the procedures given by Harris & Hopkinson (1976). Eight samples were applied to each starch gel (21.5 × 14.0 × 0.1 cm thick) by means of cotton threads, 1 cm long, placed in slots at a distance of 7 cm from the cathode (Kilgour & Godfrey, 1973; Kilgour et al., 1974). The temperature of the cooling plate was maintained at 8 °C during electrophoresis. Developer solutions were applied as agar overlays approximately 2 mm thick (final agar concentration, 6 mg ml⁻¹) except for the aminotransferase reagents which were applied on filter paper (Whatman no. 1) overlays. The overlaid plates were incubated at 37 °C in the dark until bands of a density suitable for photography were obtained. The incubation time varied from 15 to 180 min, depending on the nature and activity of the enzyme. For each enzyme examined, control plates were run on all extracts under the same electrophoretic and developmental conditions except that the main substrate was omitted. With the more complex developing reagents, additional controls were run with omission of other ingredients, such as co-substrates, activators or coupling enzymes. Samples were applied at comparable enzyme activities, being diluted when necessary in 5% sterile bovine serum albumin. Enzymes could usually be detected if used within an activity range from 0.2 to 1.0 μmol substrate transformed min⁻¹ ml⁻¹, depending on the enzyme and staining method. Where activities were very low, membrane-concentrated samples were sometimes used. Alternatively, additional material was loaded on to the cotton threads by repeated dipping and drying (V. Kilgour, personal communication): the thread was dipped in a sample, then dried on Parafilm under gentle heat from a bench lamp, this process being repeated 3 to 5 times before applying the thread to the gel. Samples were run at least twice on different occasions to ensure that results were reproducible; those with similar mobilities were also run as mixtures as a check on electrophoretic identity.

**Enzyme assays.** Activities were normally determined before electrophoresis by quantitative spectrophotometric assay at 340 nm and 30 °C using standard procedures. Assay mixtures involving NAD⁺-dependent enzymes were modified when necessary in 5% sterile bovine serum albumin. Enzymes could usually be detected if used within an activity range from 0.2 to 1.0 μmol substrate transformed min⁻¹ ml⁻¹, depending on the enzyme and staining method. Where activities were very low, membrane-concentrated samples were sometimes used. Alternatively, additional material was loaded on to the cotton threads by repeated dipping and drying (V. Kilgour, personal communication): the thread was dipped in a sample, then dried on Parafilm under gentle heat from a bench lamp, this process being repeated 3 to 5 times before applying the thread to the gel. Samples were run at least twice on different occasions to ensure that results were reproducible; those with similar mobilities were also run as mixtures as a check on electrophoretic identity.

**Protein assay.** Protein was assayed by the Lowry method using bovine serum albumin (Sigma) as standard.

**Chemicals.** ADP (sodium salt, grade III), l-alanine, l-aspartic acid, ATP (disodium salt, grade I), D-fructose 6-phosphate (disodium salt, grade I), D-glucose 6-phosphate (sodium salt, crystalline), l(+)-lactate (lithium salt, grade L-X), phosphoeno-pyruvate dihydrogenase (monocyclohexylamine salt), 2-oxoglutarate (sodium salt), D-3-phosphoglycerate (sodium salt, grade II), pyruvate (sodium salt, type II), MTT (tetrazolium salt), phenazine methosulphate (PMS) and the enzymes, glucose-6-phosphate dehydrogenase (type XV, baker's yeast, sulphate-free), glycerol-3-phosphate dehydrogenase (type I, rabbit muscle), hexokinase (type C-130, yeast), phosphoglucomutase (rabbit muscle), pyruvate kinase (type II, rabbit muscle), triosephosphate isomerase (type III, rabbit muscle) were from Sigma. NADH (disodium salt, grade II), glyceraldehyde-3-phosphate dehydrogenase [rabbit muscle, crystalline suspension in (NH₄)₂SO₄ solution], lactate dehydrogenase (horse muscle in 50% glycerol) and malate dehydrogenase (pig heart in 50% glycerol) were from Boehringer. Fructose 1,6-bisphosphate (trisodium heptahydrated salt), glucose 1-phosphate [with 1% (w/w) glucose 1,6-bisphosphate], NAD⁺ (free acid) and NADP⁺ (sodium salt) were from Wessex Biochemicals, Poole, Dorset. Other chemicals used were of the highest grade available.

**RESULTS**

Eleven enzymes were detected in extracts of *A. laidlawii* after electrophoresis. Satisfactory isoenzyme patterns with distinct banding were obtained with nine of these, namely, hexokinase, phosphoglucomutase, lactate dehydrogenase, aspartate aminotransferase, adenylate kinase, glucose-6-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase and enolase. These enzymes showed a single intensely stained band, except phosphoglycerate kinase which showed an additional weaker but well-defined band. An additional faint component close to the major band developed in a few instances with phosphoglucomutase and hexokinase. The other two enzymes detected, glucosephosphate isomerase and alanine
aminotransferase, proved to be unsatisfactory for characterization purposes because of poorly defined banding under the conditions tested.

With the two strains of *A. equifetale*, conclusive results were only obtained with phosphoglucomutase and hexokinase because of shortage of material and low enzyme activities, although in preliminary experiments adenylate kinase and pyruvate kinase were detected.
Fig. 2. Electrophoretic plates demonstrating isoenzymes of hexokinase (a), adenylate kinase (b), phosphoglucomutase (c) and lactate dehydrogenase (d) in soluble extracts of *A. laidlawii* and *A. equifetale*. Bands moved from the origin slots at the bottom towards the anode at the top. Samples, some run as mixtures, were (from left to right): (a) *A. laidlawii* strains N99, N99 plus 358, 358, PG8, PG8 plus PG9, PG9, and *A. equifetale* strains C112, N93; (b) *A. laidlawii* strains PG8, PG9, N99, 358; (c) *A. equifetale* strains C112 plus N93, N93, *A. laidlawii* strains PG8, PG9, *A. equifetale* strains C112, N93, and *A. laidlawii* strains PG8, PG9; (d) *A. laidlawii* strains PG8, PG9, N99, 358.

after electrophoresis. Electrophoretic patterns of eight of the nine enzymes examined in the various strains of *Acholeplasma* are shown in Fig. 1; the remaining enzyme, enolase, gave a result similar to that for pyruvate kinase, the mobility of the bands being approximately 1.7 times greater. All of the enzymes moved towards the anode.

*Hexokinase*. Five isoenzymes of hexokinase were detected, three in four strains of *A. laidlawii* (Fig. 1). Electrophoresis of mixtures of extracts confirmed the identity of the hexokinases of strains PG8 and PG9 and the dissimilarity in mobility of hexokinases of strains N99 and 358 (Fig. 2a). Although complete separation of this last mixture is not apparent in Fig 2(a), due to overloading, separation was obtained in two additional experi-
ments. Two other isoenzymes of hexokinase were detected in the *A. equifetale* strains C112 and N93. A faint, diffuse, fast moving band was detected in some of the samples but as this band also appeared on the control plates it could not be due to hexokinase; it was thought to be produced by the action of a reducing agent, perhaps the dithiothreitol in the lysate, on the tetrazolium salt (MTT) in the developer. The hexokinases of strains PG8 and PG9 grown with and without glucose banded in the same position.

**Phosphoglucomutase.** Three isoenzymes were detected amongst the four strains of *A. laidlawii* (Figs 1 and 2c). Those of strains PG8 and PG9 were different from each other and from those in *A. equifetale*. The isoenzymes of the two *A. equifetale* strains appeared to be identical when run as a mixture (Fig. 2c).

**Lactate dehydrogenase** (NAD+-dependent). Activity was easily detected with pyruvate as substrate using the fluorescent method. Three different patterns of distinct single bands were obtained for the four *A. laidlawii* strains (Figs 1 and 2d). The specificity of the enzyme for L(+)-lactate was confirmed by staining for activity by the formazan method in the direction lactate to pyruvate using the same electrophoresis conditions as for the fluorescent method. Activity was very low under these conditions but weak formazan bands were observed corresponding to the specific enzyme bands obtained by the fluorescent method. Additional stronger formazan bands were obtained with NAD+ and the fructose 1,6-bisphosphate added as an activator for lactate dehydrogenase; these bands were distinguished from the weaker lactate dehydrogenase bands by their continued presence on control plates with lactate omitted.

**Aminotransferase.** Concentrated samples of the *A. laidlawii* strains PG8, PG9 and N99 were examined for alanine aminotransferase and aspartate aminotransferase. Aspartate aminotransferase differed in all three (Fig. 1). The results for alanine aminotransferase were inconclusive. The enzyme was only detected satisfactorily in strain N99 as a distinct single band, weak ill-defined patches being obtained with strains PG8 and PG9. Additional bands occurred which were not due to aminotransferase activity, as shown by control plates without substrate. These bands were probably formed by a NADH dehydrogenase detected in the controls of the spectrophotometric assays where NADH was a reactant.

**Adenylate kinase.** Two isoenzymes of adenylate kinase were present in *A. laidlawii*, one in the reference strains and the other in the equine strains (Figs. 1 and 2b). The reference strains gave similar results when grown without glucose.

**Glucose-6-phosphate dehydrogenase.** As with adenylate kinase, two isoenzymes with differing mobilities were detected in *A. laidlawii*, one in the reference strains and the other in the equine strains (Fig. 1). The substitution of fructose 6-phosphate for glucose 6-phosphate in the staining system gave bands similar to those for glucose 6-phosphate dehydrogenase. This was probably due either to trace impurities of glucose 6-phosphate or to the conversion in situ of fructose 6-phosphate to glucose 6-phosphate by glucosephosphate isomerase which had spread across the plate and into the area occupied by the glucose-6-phosphate dehydrogenase bands.

**Phosphoglycerate kinase.** For each of the *A. laidlawii* strains this enzyme gave a pattern consisting of a major band plus a minor band of slightly higher mobility (Fig. 1). The isoenzyme for strain 358 differed from that common to the other three strains.

**Pyruvate kinase.** The pyruvate kinase bands of all four strains of *A. laidlawii* had identical mobilities, as shown by a fluorescent staining method specific for this enzyme (Fig. 1). Using the non-specific formazan staining method for pyruvate kinase, which also stains for adenylate kinase, samples of strains PG8 and PG9 grown without glucose again showed the same bands. Preliminary experiments with the two *A. equifetale* strains gave indications of different isoenzymes.

**Enolase.** Identical single bands were observed in the four *A. laidlawii* strains.

**Glucosephosphate isomerase.** Although, on assay, the activity of this enzyme in all the samples was very high relative to the other enzymes tested (see below), the zymograms
Isoenzymes in Acholeplasma obtained after electrophoresis were unsatisfactory. The enzyme stained as diffuse columns extending varying distances from the origin depending on the strain thus suggesting that strain differences occur.

Fructose 1,6-bisphosphate dehydrogenase. As mentioned above, additional distinct fomazan bands due to a dehydrogenase utilizing NAD+ and fructose 1,6-bisphosphate were detected in control plates in the lactate dehydrogenase experiments where fructose 1,6-bisphosphate had been added as a possible activator. However, when NADP+ was used as a coenzyme with fructose 1,6-bisphosphate as substrate, two sets of bands were obtained, one set being due to the so-called 'fructose-1,6-bisphosphate dehydrogenase'. The other set was identical to the glucose-6-phosphate dehydrogenase pattern and was probably caused by the presence of either glucose 6-phosphate or fructose 6-phosphate as trace impurities in the fructose 1,6-bisphosphate, the fructose 6-phosphate being converted to glucose 6-phosphate by phosphoglucose isomerase (see glucose-6-phosphate dehydrogenase results). Further experiments confirmed that this 'fructose-1,6-bisphosphate dehydrogenase' utilized either NAD+ or NADP+ in its reaction. This enzyme was investigated only in the four A. laidlawii strains. Strains PG8 and PG9 showed single bands of almost identical mobility, faster than the band obtained for N99. No conclusions could be drawn from the weak, indistinct band given by strain 358.

Other enzymes. Malate dehydrogenase, 'malic enzyme', isocitrate dehydrogenase (NADP+) and phosphogluconate dehydrogenase (decarboxylating) were not detected in the four strains of A. laidlawii. Strains of A. equinale were not tested for these enzymes.

Enzyme assays. The ranges of specific enzyme activities [expressed as μmol substrate transformed min⁻¹ (mg protein)⁻¹] obtained with the four A. laidlawii strains were as follows: alanine aminotransferase, trace to 0·1; aspartate aminotransferase, trace to 0·6; glucose-6-phosphate dehydrogenase, 0·1 to 0·5; glucosephosphate isomerase, 12·2 to 30·3; hexokinase, 0·4 to 0·9; lactate dehydrogenase, 0·7 to 2·3; phosphoglucomutase, 0·7 to 2·8; NADH dehydrogenase, 0·1 to 5·2. Isocitrate dehydrogenase (NADP+), malate dehydrogenase, 'malic enzyme' and phosphogluconate dehydrogenase (decarboxylating) were undetectable by assay which corroborated the negative electrophoretic results.

DISCUSSION

Isoenzyme electrophoretic analysis can be easily applied to the soluble enzymes of mycoplasmas and has considerable potential as a taxonomic tool for subspecific differentiation. Previous work has been restricted to the examination of only a few enzymes compared with the nine enzymes used in this investigation.

Neimark & Lemcke (1972) demonstrated multiple forms of NAD+ -dependent l(+) -lactate dehydrogenase in six mycoplasmas which included one Acholeplasma species, A. axanthum, and obtained confirmatory evidence for assigning an unclassified strain as Mycoplasma mycoides var. mycoides. Allsopp & Matthews (1975) compared the same enzyme in ten strains of M. mycoides var. mycoides but could not detect any intraspecific differences, and further studies using isoelectric focusing gave equivocal results. Delisle (1977) detected four multiple banded patterns of urease amongst five strains of Ureaplasma urealyticum. The patterns were useful for distinguishing strains as they remained constant for organisms harvested at different times during the exponential phase of growth and after repeated subculture. Two forms of arginine deiminase were present in a strain of M. arthritidis at different stages in the growth cycle (Weickmann & Fahrney, 1977) and it was postulated that the stationary phase form of the enzyme arose from the introduction of a new chromophoric group into the earlier exponential phase form.

In the present work, all the strains were cloned and variations related to the growth phase were avoided by examining the organism in the exponential phase when the pH had fallen
by only 0.5 units. No differences were observed in the patterns of isoenzymes when compared for organisms grown with or without glucose.

The enzymes which showed the greatest variability in the two species of *Acholeplasma* examined were hexokinase and phosphoglucomutase. Isoenzymes of these revealed specific and intraspecific differences. Likewise, lactate dehydrogenase, aspartate aminotransferase, adenylate kinase, glucose-6-phosphate dehydrogenase and phosphoglycerate kinase could provide useful markers, since these enzymes showed intraspecific differences amongst the *A. laidlawii* strains and could be demonstrated by a relatively simple staining system. The isoenzymes of adenylate kinase and glucose-6-phosphate dehydrogenase divided the *A. laidlawii* strains according to their source—sewage and the equine respiratory tract; such isoenzymes might function as epidemiological markers for tracing distinct populations among pathogenic mycoplasmas.

Enolase and pyruvate kinase did not show intraspecific differences, but could be useful in species differentiation as alternatives to serological tests or to the electrophoretic analysis of cell proteins. Lack of material precluded screening of *A. equifetale* for lactate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphoglycerate kinase and enolase, but these were probably present since other glycolytic enzymes were found in this species. The results with glucosephosphate isomerase indicated possible strain differences and further attempts to find the optimal electrophoretic conditions for resolution may be rewarding.

Aspartate aminotransferase was one of the enzymes useful for detecting strain variability in *A. laidlawii*, but activity was low in most extracts and concentration methods had to be applied. Alanine aminotransferase activity was even lower and difficult to detect electrophoretically even after concentration. However, both types of aminotransferase could be detected in *A. laidlawii*, albeit at a low level, in some of the strains. Rodwell (1960) and Razin & Cohen (1963) failed to find aminotransferase activity in *M. mycoides* or *A. laidlawii*, respectively, although Gill (1962) reported a transamination system between glutamic and aspartic acid in *M. gallisepticum* strain A5969.

Some of the other enzymes demonstrated have not been identified previously in mycoplasmas. These were adenylate kinase and three of the enzymes in the last part of the Embden–Meyerhof pathway—phosphoglycerate kinase, enolase and pyruvate kinase. Smith (1971) postulated the presence of enolase in *M. mycoides* on the basis of the fluoride inhibition of glucose fermentation.

An NADP⁺-dependent glucose-6-phosphate dehydrogenase was demonstrated in all four *A. laidlawii* strains, suggesting the possible presence of the hexose-monophosphate shunt, although the expected sequent enzyme, phosphogluconate dehydrogenase, was not detected. Castrejon-Diez et al. (1963) concluded that the hexose-monophosphate shunt was absent from Type A but active in the Adler strain. Additional evidence was the absence of glucose-6-phosphate dehydrogenase in Type A and its presence in the Adler strain, together with two other enzymes of the pathway—ribose-5-phosphate isomerase and transketolase. Glucose-6-phosphate dehydrogenase was, however, subsequently demonstrated by Pollack et al. (1965) in *A. laidlawii* Type A and also in Type B.

In accordance with the spectrophotometric findings of Neimark & Tung (1973), the NAD⁺-dependent *l*(+)-lactate dehydrogenase of the *A. laidlawii* strains stained strongly in the direction of lactate formation but only weakly in the reverse direction. This reaction was in marked contrast to the behaviour of the NAD⁺-dependent *l*(+)-lactate dehydrogenase isoenzymes demonstrated by Neimark & Lemcke (1972) in other mycoplasmas, including *A. axanathum*, which stained strongly in the reverse direction, i.e. towards pyruvate formation. The failure to detect malate dehydrogenase and isocitrate dehydrogenase (NADP⁺) either by assay or after electrophoresis correlates with the observations of Tourtellotte & Jacobs (1960) and Neimark & Pickett (1960) who found no evidence for the existence of the tricarboxylic acid cycle in several mycoplasmas including *A. laidlawii*. 
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The majority of the enzymes examined appeared as a single form in each strain and, apart from the possibility that these single bands could be a mixture of heterogeneous molecules with the same electrophoretic mobility, it is probable that they are coded for by a single gene locus. With hexokinase and phosphoglucomutase, the faint subsidiary bands observed in close proximity to the main band were probably products from mild degradation of the enzyme during extraction and storage. Phosphoglycerate kinase, however, showed a more pronounced secondary band and is possibly more easily degraded or dissociated than the other enzymes; alternatively it could be coded by more than one gene locus.

Isoenzyme analysis by electrophoresis is being used increasingly to resolve taxonomic problems in other groups of micro-organisms because it frequently reveals genetically determined differences in the primary protein structure of enzymes. It has the advantages of technical simplicity and easy interpretation over electrophoretic analysis of cell proteins and nucleic acid hybridization techniques which also reflect differences in genetic constitution. This investigation was essentially exploratory, but it has shown for the first time that a number of enzymes occur as distinct electrophoretic forms of potential value for distinguishing strains of Acholeplasma, both at the specific and subspecific levels.

This work was supported by the Ministry of Overseas Development (S.M.L., C.M.S. and J.M.G.). We thank Dr D. G. Godfrey and Dr V. Kilgour for their helpful advice and criticism, and Miss Militsa Philippou for excellent technical assistance.

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