Presence of Anaplerotic Reactions and Transamination, and the Absence of the Tricarboxylic Acid Cycle in Mollicutes

By JOHN T. MANOLUKAS,¹ MICHAEL F. BARILE,²
DONNA K. F. CHANDLER² AND J. DENNIS POLLACK¹*

¹ Department of Medical Microbiology and Immunology, The Ohio State University, Columbus, Ohio 43210, USA

² Division of Bacterial Products, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland 20205, USA

(Received 1 July 1987; revised 12 October 1987)

Cell extracts of the fermentative Mollicutes *Acholeplasma laidlawii* B-PG9, *Acholeplasma morum* S2, *Mycoplasma capricolum* 14, *Mycoplasma gallisepticum* S6, *Mycoplasma pneumoniae* FH, *Mycoplasma hyopneumoniae* J and *M. genitalium* G-37, and the non-fermentative *Mycoplasma hominis* PG-21, *Mycoplasma hominis* 1620 and *Mycoplasma bovis* PG-11 were examined for 39 cytoplasmic enzyme activities associated with the tricarboxylic acid (TCA) cycle, transamination, anaplerotic reactions and other enzyme activities at the pyruvate locus. Malate dehydrogenase (EC 4.2.1.2) was the only TCA-cycle-associated enzyme activity detected and it was found only in the eight *Mycoplasma* species. Aspartate aminotransferase (EC 2.6.1.1) activity was detected in all Mollicutes tested except *M. gallisepticum* S6. Malate synthetase (EC 4.1.3.2) activity, in the direction of malate formation, was found in the eight *Mycoplasma* species, but not in any of the *Acholeplasma* species. Phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) was detected in the direction of oxaloacetate (OAA) formation in both *Acholeplasma* species, but not in any of the *Mycoplasma* species. Pyruvate carboxylase (EC 6.4.1.1), pyruvate kinase (EC 2.7.1.40), pyruvate dehydrogenase (EC 1.2.4.1) and lactate dehydrogenase (EC 1.1.1.27) activities were found in all ten Mollicutes tested. No activities were detected in any of the ten Mollicutes for aspartase (EC 4.3.1.1), malic enzyme (EC 1.1.1.40), PEP carboxytransphosphorylase (EC 4.1.1.38), PEP carboxykinase (EC 4.1.1.32) or pyruvate orthophosphate dikinase (EC 2.7.9.1). In these TCA-cycle-deficient Mollicutes the pyruvate–OAA locus may be a point of linkage for the carbons of glycolysis, lipid synthesis, nucleic acid synthesis and certain amino acids. CO₂ fixation appears obligatory in the *Acholeplasma* species and either CO₂ fixation or malate synthesis appears obligatory in the *Mycoplasma* species.

INTRODUCTION

The Mollicutes, a class of prokaryotic organisms characterized by the lack of a cell wall, can be divided into fermentative and non-fermentative groups based on their carbohydrate metabolism (Smith, 1971). The fermentative group produces acid from carbohydrates such as glucose (Tourtellotte & Jacobs, 1960). In these fermentative Mollicutes, glucose is oxidized to acetate and CO₂, and lactate is the major end product of glycolysis (Gill, 1960; Neimark & Pickett, 1960; Castrejon-Diez *et al.*, 1963; Smith, 1971; Beaman & Pollack, 1981; Reinards *et al.*, 1981). The non-fermentative group does not significantly lower the pH of the culture medium and some of these organisms are known to oxidize fatty acids and alcohols (Lynn, 1960; VanDemark & Smith, 1965; Beaman & Pollack, 1983).

Abbreviations: TCA cycle, tricarboxylic acid cycle; PEP, phosphoenolpyruvate; OAA, oxaloacetate; MDH, malate dehydrogenase; PFK, phosphofructokinase.

Apart from the report of VanDemark & Smith (1964*b*), there is little additional evidence for the presence of the tricarboxylic acid (TCA) cycle in non-fermentative Mollicutes (Holmes & Pirie, 1932; Leece & Morton, 1954; Tourtellotte & Jacobs, 1960). The non-fermentative strain previously designated *Mycoplasma hominis* 07, now known as *Mycoplasma arthritidis* 07, was reported to be capable of oxidizing TCA intermediates (VanDemark & Smith, 1964*b*). The presence of the TCA cycle in the non-fermentative 07 strain was compatible with the observations that the same strain had cytochrome pigments and evidence for the presence of oxidative phosphorylation activity (VanDemark & Smith, 1964*a*). However, the relationship was diminished by our contrary finding that cytochrome pigments are absent from the 07 strain and from other Mollicutes (Pollack *et al.*, 1981), and that quinones are present only in trace amounts in these organisms (Hollaender *et al.*, 1977).

Parenthetically, a Fe/Cu flavoprotein (FMN) dehydrogenase has been found and purified in *Acholeplasma laidlawii* (Reinards *et al.*, 1981) and a similar flavoprotein (FAD) dehydrogenase has been purified from *Mycoplasma capricolum* 14 (Klomkes *et al.*, 1985). Although the hypothesis is untested, these dehydrogenases might act as electron acceptors leading to the conservation of energy in a manner analogous to the activity of a Site-I-like locus of classical electron transport in mitochondria, i.e., there may be some limited form of oxidative phosphorylation in these Mollicutes.

The fermentative *Mycoplasma mycoides* subsp. *mycoides* cannot metabolize citrate, 2-oxoglutarate, succinate or fumarate (Rodwell & Rodwell, 1954) and strains of the fermentative *A. laidlawii* lack malate dehydrogenase (MDH) (Lanham *et al.*, 1980). The absence of such activities supported the concept that the fermentative Mollicutes may lack the TCA cycle while the non-fermentative Mollicutes, at least *M. arthritidis* (*M. hominis*) 07, have the TCA cycle. More recently, the reports of the presence of MDH activity in some fermentative Mollicutes suggests that at least part of the TCA cycle might be present in such organisms (Salih *et al.*, 1983; Cocks *et al.*, 1985).

In preliminary studies we were unable to confirm the presence of the TCA cycle in other strains of *Mycoplasma hominis*, and decided to reinvestigate such metabolism in Mollicutes. In this study, we investigated enzyme activities associated with the TCA cycle, transamination, anaplerotic and other enzyme activities at the pyruvate locus in ten Mollicutes. The Mollicutes we studied were the fermentative *Acholeplasma laidlawii* B-PG9, *Acholeplasma morum* S2, *Mycoplasma capricolum* 14, *Mycoplasma gallisepticum* S6, *Mycoplasma pneumoniae* FH, *Mycoplasma hyopneumoniae* J, *M. genitalium* G-37 and the non-fermentative *Mycoplasma hominis* PG-21, *Mycoplasma hominis* 1620 and *Mycoplasma bovis* PG-11.

METHODS

Organisms. Ten Mollicutes representing acholeplasmas and both fermentative and non-fermentative mycoplasmas were selected. From our stock collections we chose two acholeplasmas – *Acholeplasma laidlawii* B-PG9 and *Acholeplasma morum* S2 – the fermentative mycoplasmas *Mycoplasma capricolum* 14, *Mycoplasma gallisepticum* S6 and *Mycoplasma pneumoniae* FH, and the non-fermentative *Mycoplasma hominis* PG-21, *Mycoplasma hominis* 1620 and *Mycoplasma bovis* PG-11. The fermentative *Mycoplasma genitalium* G-37 was obtained from V. V. Tryon, University of Texas Health Science Center at San Antonio, San Antonio, Tx., USA. The fermentative *Mycoplasma hyopneumoniae* J was obtained from R. F. Ross, Iowa State University, Ames, Ia., USA. *Escherichia coli* ATCC 29522, *Streptococcus faecium* ATCC 6051 and a recent isolate of *Pseudomonas aeruginosa*, identified by University Hospital, The Ohio State University, USA, were also examined.

Media and growth conditions. Unless indicated organisms were grown statically in 4–6 cm deep layers in our modification of Edward medium at 37 °C until mid-exponential phase (1–4 d) (Beaman & Pollack, 1981). For the growth of the fermentative Mollicutes, the media were supplemented with 0–0.5% heat inactivated horse serum for *A. laidlawii* and *A. morum*, and with 5% (v/v) serum for *M. capricolum* and *M. gallisepticum*. *M. pneumoniae* and *M. bovis* were grown in a similar medium containing 0.002% phenol red, 5% (v/v) fresh yeast extract (Flow Laboratories) instead of powdered yeast extract, and 10% (v/v) heat inactivated horse serum. *M. hominis* strains were grown in media similar to that used for *M. pneumoniae* but supplemented with 0.15% arginine. HCl, while the fresh yeast extract concentration was reduced to 1% and the pH was 6.8–7.0. *M. genitalium* was grown in

Hayflick medium (Lipman *et al.*, 1969) supplemented with 20% (v/v) heat inactivated horse serum and 0.04% phenol red. *M. hyopneumoniae* was grown at 36 °C in Friis broth culture (Friis, 1975) containing 10% (v/v) acid adjusted swine serum (Slavik & Switzer, 1972). *M. hyopneumoniae* and *A. laidlawii* were also grown with constant shaking (approximately 100 r.p.m., 10 cm stroke, model 3590, Labline Instruments). *M. hominis* 1620 was grown with and without sparging at a rate of 16 l sterile air h⁻¹ (1 medium)⁻¹ for 1–2 d. *M. pneumoniae* was grown attached to plastic 150 cm² tissue culture flasks. *M. pneumoniae* organisms were harvested by pouring off the culture medium, scraping the attached organisms into kappa buffer (Pollack *et al.*, 1970) and centrifuging at 12 000 g for 30 min at 4 °C.

Preparation of cell extracts. Cell preparations were made essentially as previously described (Pollack, 1975, 1983). Cells were collected and washed by centrifugation. Washed cells were lysed by osmotic shock or by explosive decompression in a Parr-Bomb. Cells lysates were fractionated by centrifugation and then dialysed.

Enzyme assays. In cytoplasmic fractions from each of 10 *Mollicutes*, 39 enzyme activities comprising or associated with the TCA cycle, transamination, anaplerotic and other activities at the pyruvate locus were assayed spectrophotometrically (model 260, Gilford). Reaction mixtures contained 2.3×10^{-2} – 9.1×10^2 µg protein ml⁻¹ and the assays were done aerobically and in some cases anaerobically in nitrogen or helium. When no activity was detected with our cytoplasmic extracts at a reaction mixture concentration of 70% (v/v), we added approximately 0.001 U of commercially available purified enzyme (Sigma). This was a test for the sensitivity and appropriateness of our assay conditions, and the presence of inhibitors in the *Mollicute* cytoplasmic extract. HEPES (Research Organics) was the only buffer used in our studies. Protein was determined using the G-250 dye reagent formulated by Bio-Rad. Pyrophosphate (PP_i) was assayed using a pyrophosphate assay kit (Sigma) according to the manufacturer's directions.

Enzymes of the TCA cycle. (i) Citrate synthase (EC 4.1.3.7) was assayed by both the methods of Srere (1969) and Stitt (1983a). (ii) Aconitase (EC 4.2.1.3) was assayed by a modification of the method of Goldberg & Ellis (1983). The reaction mixture (1.0 ml) contained 100 mM-HEPES/NaOH (pH 7.4), 1.7 mM-MnCl₂, 0.15 mM-NADP⁺, 1 U isocitrate dehydrogenase and cell extract. The reaction was started with 5 mM-citric acid. (iii) Isocitrate dehydrogenase (EC 1.1.1.42) was assayed by the method of Goldberg & Ellis (1983). (iv) 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was assayed by the method of Reed & Mukherjee (1963). (v) Succinate synthase (EC 6.2.1.5) was assayed by the method of Bridger *et al.* (1969). (vi) Succinate dehydrogenase (EC 1.3.99.1) was assayed by the methods of Reddy & Weber (1986). (vii) Fumarate reductase (EC 1.3.1.6) was assayed by the methods of George & Smibert (1982). (viii) Fumarase (EC 4.2.1.2) was assayed by the procedure of Stitt (1983b). (ix) Malate dehydrogenase (EC 1.1.1.37) was assayed by the method of Yoshida (1969).

Transaminase, anaplerotic and other activities at the pyruvate locus. (i) Aspartate aminotransferase (EC 2.6.1.1) was assayed by the procedure of Rej & Horder (1983) at 37 °C. (ii) Aspartase (EC 4.1.3.2) was assayed by the procedure of Williams & Lartigue (1969). (iii) Malic enzyme (EC 1.1.1.40) was assayed according to the method of Hsu & Lardy (1969). (iv) Phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) was assayed according to the method of Smith (1968). We also assayed for CO₂ fixation using 0.4 µCi (14.8 kBq) NaH¹⁴CO₃ in each reaction mixture [specific activity 0.1 mCi mmol⁻¹ (3.7 MBq mmol⁻¹); Research Products International], by the method of Cox & Baugh (1976). (v) PEP carboxytransphosphorylase (EC 4.1.1.38) and PEP carboxykinase (EC 4.1.1.32) were assayed by a modification of the procedure of Smith (1968). The basic reaction mixture (1.0 ml) contained 100 mM-HEPES/NaOH (pH 7.4), 6 mM-PEP, 5 mM-MgCl₂ or MnCl₂, 0.1 U malate dehydrogenase (MDH), 0.15 mM-NADH and cell extract. For the PEP carboxytransphosphorylase assay, 5 mM-KH₂PO₄ (P_i) was also added and for the PEP carboxykinase assay either 4 mM-ADP or 4 mM-GDP was added and the reactions were started by the addition of 10 mM-NaHCO₃. (vi) Pyruvate carboxylase (EC 6.4.1.1) was assayed by the method of Holdsworth & Bruck (1977). Also, ATP formed by this reaction was measured directly by following the formation of NADPH at 340 nm in a mixture (1.0 ml) containing 100 mM-HEPES/NaOH (pH 7.4), 1.8 mM-glucose, 0.1 U hexokinase, 10 mM-glucose 6-phosphate, 0.1 U glucose-6-phosphate dehydrogenase, 15 mM-NADP⁺, 4 mM-ADP, 140 mM-oxaloacetate (OAA), 5 mM-KH₂PO₄ (P_i) and cytoplasmic extract. (vii) Pyruvate dehydrogenase (EC 1.2.4.1) was assayed by the procedure of McGarrity *et al.* (1984). Pyruvate kinase (EC 2.7.1.40) was assayed by the procedure of Bucher & Pfeleiderer (1955). (viii) Pyruvate orthophosphate dikinase (EC 2.7.9.1) was assayed by the procedure of Ernst *et al.* (1986). (ix) Lactate dehydrogenase (EC 1.1.1.27) was assayed by the procedure of Kornberg (1955). (x) Malate synthase (EC 4.1.3.2) was assayed by the procedure of Dixon & Kornberg (1962).

Statistical analysis. Each enzyme activity in each batch of cells was assayed at three to five concentrations of cell extract to determine the maximum specific activity (rate). The mean rates and SD values we report were calculated from the individual rates obtained from each of six to fifteen different batches of cells for *A. laidlawii* B-PG9, *A. morum* S2, *M. capricolum* 14 and *M. gallisepticum* S6, and from three batches of *M. hyopneumoniae* J, *M. pneumoniae* FH, *M. hominis* 1620 and *M. bovis genitalium* PG-11. Two batches of *M. hominis* PG-21 and one batch of *M. genitalium* G-37 were examined.

Table 1. *Enzyme activities in extracts of the Acholeplasma and non-fermentative Mycoplasma species*Enzyme activity is expressed as nmol product synthesized min⁻¹ (mg protein)⁻¹ (mean $\pm$ SD).

Enzyme	Reaction no.*	<i>A. laidlawii</i> †	<i>A. morum</i> †	<i>M. bovigenitalium</i> †	<i>M. hominis</i> 1620†	<i>M. hominis</i> PG-21‡
Malate dehydrogenase	14	ND	ND	212 $\pm$ 3	111 $\pm$ 9	127
	15	ND	ND	925 $\pm$ 6	713 $\pm$ 15	640
Aspartate aminotransferase	16	26.6 $\pm$ 0.3	150 $\pm$ 10	826 $\pm$ 4	789 $\pm$ 9	650
	17	426 $\pm$ 12	430 $\pm$ 25	53 $\pm$ 2	19 $\pm$ 1	12
Malate synthase	20	ND	ND	412 $\pm$ 14	214 $\pm$ 2	423
PEP carboxylase	24	880 $\pm$ 49	840 $\pm$ 50	ND	ND	ND
Pyruvate carboxylase	30	2.9 $\pm$ 0.1	7.2 $\pm$ 0.8	4500 $\pm$ 36	1150 $\pm$ 14	1290
	31	0.30 $\pm$ 0.02	0.90 $\pm$ 0.03	25 $\pm$ 1	16 $\pm$ 1	31
Pyruvate dehydrogenase	32	13.8 $\pm$ 0.2	10.3 $\pm$ 0.2	11.6 $\pm$ 0.11	5.3 $\pm$ 0.8	17
	33	1.9 $\pm$ 0.03	1.9 $\pm$ 0.1	2.50 $\pm$ 0.03	1.30 $\pm$ 0.02	2
Pyruvate kinase	34	0.9 $\pm$ 0.1	1.9 $\pm$ 0.3	9.2 $\pm$ 0.1	2.1 $\pm$ 0.01	3
	35	5.6 $\pm$ 0.3	8.9 $\pm$ 0.5	102 $\pm$ 2	73 $\pm$ 1	88
Lactate dehydrogenase	38	308 $\pm$ 10	144 $\pm$ 7	756 $\pm$ 4	94 $\pm$ 6	78
	39	16 $\pm$ 1	938 $\pm$ 27	1.3 $\pm$ 0.03	3.2 $\pm$ 0.02	2

ND, No activity was ever detected; we could detect 1 mU enzyme activity in samples purchased from Sigma.

* Corresponds to the reaction no. in Figs 1 and 2.

† No. of batches of each organism that was tested for enzyme activity was three to fifteen.

‡ No. of batches of each organism that was tested for enzyme activity was two.

Table 2. *Enzyme activities in extracts of fermentative Mycoplasma species*Enzyme activity is expressed as nmol product synthesized min⁻¹ (mg protein)⁻¹ (mean $\pm$ SD).

Enzyme	Reaction no.*	<i>M. gallisepticum</i> †	<i>M. capricolum</i> †	<i>M. pneumoniae</i> †	<i>M. hyopneumoniae</i> †	<i>M. genitalium</i> ‡
Malate dehydrogenase	14	55 $\pm$ 5	552 $\pm$ 9	116 $\pm$ 2	466 $\pm$ 26	49
	15	412 $\pm$ 44	1210 $\pm$ 40	889 $\pm$ 10	1150 $\pm$ 20	452
Aspartate aminotransferase	16	ND	1250 $\pm$ 40	1010 $\pm$ 12	142 $\pm$ 8	51
	17	ND	73 $\pm$ 7	37 $\pm$ 2	22 $\pm$ 1	5
Malate synthase	20	202 $\pm$ 6	428 $\pm$ 9	765 $\pm$ 7	121 $\pm$ 2	23
PEP carboxylase	24	ND	ND	ND	ND	ND
Pyruvate carboxylase	30	2340 $\pm$ 20	7080 $\pm$ 20	2300 $\pm$ 70	1490 $\pm$ 7	526
	31	22 $\pm$ 2	16 $\pm$ 1	11 $\pm$ 0.9	9.5 $\pm$ 0.2	2
Pyruvate dehydrogenase	32	9 $\pm$ 1	10 $\pm$ 0.3	2.6 $\pm$ 0.03	16 $\pm$ 1	22
	33	2 $\pm$ 0.2	2 $\pm$ 0.2	2.3 $\pm$ 0.1	1.4 $\pm$ 0.01	5
Pyruvate kinase	34	2 $\pm$ 0.2	1.9 $\pm$ 0.03	3 $\pm$ 0.1	5 $\pm$ 0.4	12
	35	20 $\pm$ 2	106 $\pm$ 8	95 $\pm$ 2	95 $\pm$ 1	300
Lactate dehydrogenase	38	89 $\pm$ 8	59 $\pm$ 6	65 $\pm$ 1.65	217 $\pm$ 2	342
	39	1.4 $\pm$ 0.1	1 $\pm$ 0.1	1.2 $\pm$ 0.1	4.3 $\pm$ 0.1	6

ND, No activity was ever detected; we could detect 1 mU enzyme activity in samples purchased from Sigma.

* Corresponds to the reaction no. in Fig. 2.

† No. of batches of each organism that was tested for enzyme activity was three to fifteen.

‡ No. of batches of each organism that was tested for enzyme activity was one.

Fig. 1. Schematic diagram of the enzyme activities and pathways detected in extracts of *A. laidlawii* B-PG9 and *A. morum* S2. The enzyme assays and abbreviations are described in Methods. The reaction nos refer to the same enzyme reactions numbered and described in Table 1. The lines with a pair of diagonal slashes represent reaction sequences presumably present but not investigated in this study.

RESULTS

Enzyme activities associated with the TCA cycle

In the cytoplasmic extracts from both *Acholeplasma* species we detected no enzyme activities associated with the TCA cycle. All *Mycoplasma* species extracts contained MDH activity (Tables 1 and 2, reactions 14 and 15), but lacked any other activity associated with the TCA cycle. As a test for the possible inhibition of enzyme expression by our enriched medium *E. coli*, *P. aeruginosa* and *S. faecium* were grown under the same conditions as the Mollicutes. With the exception of citrate synthetase in *Streptococcus faecium*, we detected all nine of the enzyme activities of the TCA cycle in these three bacteria. As a test for the effects of aeration on enzyme activities, *A. laidlawii* and *M. hominis* 1620 were both grown statically and also with either shaking or sparging. We found no difference in the enzyme patterns found in either Mollicute whether grown statically or with aeration.

Transamination activity

The results of our assay for transaminase activity are shown in Tables 1 and 2 and in Figs 1 and 2 (reactions 16 and 17). With the exception of *M. gallisepticum* S6, all of the Mollicutes tested possessed aspartate aminotransferase activity in both directions.

Fig. 2. Schematic diagram of the enzyme activities and pathways detected in extracts of *M. capricolum* 14, *M. gallisepticum* S6, *M. pneumoniae* FH, *M. genitalium* G-37, *M. hyopneumoniae* J, *M. hominis* PG-21, *M. hominis* 1620 and *M. bovis genitalium* PG-11. The enzyme assays and abbreviations are described in Methods. The reaction nos refer to the same enzyme reactions numbered and described in Tables 1 and 2. The dashed lines at the aspartate aminotransferase locus (reactions 16 and 17) indicate that the reactions are present in all organisms except *M. gallisepticum*. The lines with a pair of diagonal slashes represent reaction sequences presumably present but not investigated in this study.

Anaplerotic and other activities at the pyruvate locus

No aspartase, malic enzyme, PEP carboxytransphosphorylase, PEP carboxykinase or pyruvate orthophosphate dikinase activities were detected in any of the Mollicutes tested.

PEP carboxylase in the direction of OAA formation (Table 1 and Fig. 1, reaction 24) was detected in both of the *Acholeplasma* species, but was not detected in either the fermentative or non-fermentative *Mycoplasma* species. This indicated that the *Acholeplasma* species apparently fixed CO₂ by converting PEP and CO₂ to OAA. This occurred without the addition of P_i or ADP and without the formation of ATP or PP_i, presumably as a result of PEP carboxylase activity, as neither PEP carboxytransphosphorylase or PEP carboxykinase were detectable.

We obtained more direct evidence for CO₂ fixation in *A. laidlawii*. Extracts, in the presence of MnCl₂ and PEP, could incorporate NaH¹⁴CO₃ radioactivity into material which was not volatilized from solutions of pH ≤ 2.0. A radioactive dinitrophenylhydrazine derivative of this material co-migrated with the dinitrophenylhydrazone of OAA: thus OAA was synthesized in our PEP carboxylase reaction (unpublished data). We found, as did Beaman & Pollack (1984),

that the PEP carboxylase activity in extracts from five different cell batches of *A. laidlawii* was 8.25 ± 0.87 nmol OAA synthesized min^{-1} (mg protein^{-1}).

All the *Mycoplasma* species tested contained malate synthase activity in the direction of malate formation (Tables 1 and 2, Fig. 2, reaction 20). In the *Acholeplasma* species no malate synthase activity was detected. No activity was detected in the direction of glyoxalate formation in any of the Mollicutes.

The extracts from all ten Mollicutes had pyruvate carboxylase activity in the direction of OAA formation. In the reverse direction of this reaction, where ATP is synthesized, we measured ATP synthesis directly and found it (Tables 1 and 2, Figs 1 and 2, reactions 30 and 31). All ten Mollicutes had pyruvate kinase activity in both directions and lactate dehydrogenase activity in both directions (Tables 1 and 2, Figs 1 and 2, reactions 34, 35, 38 and 39).

A. laidlawii and *M. gallisepticum* extracts have pyruvate dehydrogenase activity (McGarritty *et al.*, 1984). All other Mollicutes we tested also contained this enzyme activity (Tables 1 and 2, Figs 1 and 2, reactions 32 and 33).

DISCUSSION

Our data corroborates the view that the fermentative Mollicutes lack the complete TCA cycle and indicates that the non-fermentative Mollicutes also lack a complete TCA cycle. MDH was the only detectable activity associated with the TCA cycle and was found only in the *Mycoplasma* species, whether fermentative or non-fermentative.

The inability to detect MDH activity in *Acholeplasma* species is consistent with the findings of Lanham *et al.* (1980) who, using isoenzyme electrophoretic analysis, were unable to detect MDH activity. However, Salih *et al.* (1983) detected weak MDH activity in *A. laidlawii* by the same technique; they concluded that the low activity was due to enzyme instability. This may account for our inability to detect MDH activity in *A. laidlawii*.

Aspartate aminotransferase activity was not found in *M. mycoides* subsp. *mycoides* (Rodwell, 1960), but has been reported in *A. laidlawii* (Lanham *et al.*, 1980). However, Razin (1963) failed to detect this activity in *A. laidlawii*. We detected aspartate aminotransferase activity in all Mollicutes tested except *M. gallisepticum*; this latter observation is in conflict with Gill (1960). As the enzyme might be unstable and destroyed during preparation of the cytoplasmic extract, we assayed enzyme activity before and after ultracentrifugation and dialysis, but still failed to detect activity. Though malate synthase activity was reported absent in non-fermentative *M. arthritidis* (*M. hominis*) 07 (VanDemark & Smith, 1964b), we detected malate synthase activity in both non-fermentative and fermentative *Mycoplasma* species but only in the direction of malate synthesis.

Of the anaplerotic reactions tested, pyruvate carboxylase was found in all ten Mollicutes and PEP carboxylase in both *Acholeplasma* species but not in any *Mycoplasma* species. It was surprising not to find either PEP carboxytransphosphorylase activity or pyruvate orthophosphate dikinase activity in *A. laidlawii* or *A. morum* since these organisms have been reported to possess a PP_i -dependent phosphofructokinase (PFK) (Pollack & Williams, 1986), and the former activities were reported to be present in organisms with a PP_i -dependent PFK (Wood *et al.*, 1977; Tryon & Pollack, 1984).

Acholeplasma and *Mycoplasma* species each have two paths from PEP to OAA. One path, present in both genera, proceeds from PEP to pyruvate via pyruvate kinase with a gain of one ATP, then from pyruvate to OAA via pyruvate carboxylase with a loss of one ATP (Figs 1 and 2, reactions 35 and 30). The *Acholeplasma* species have an alternate path to OAA directly from PEP without an intervening pyruvate and the concomitant gain and loss of ATP. This alternate carboxylation path proceeds via PEP carboxylase to OAA (Fig. 1, reaction 24). Therefore, neither path from PEP to OAA in *Acholeplasma* species yields net ATP. The two *acholeplasmas* having pyruvate dehydrogenase, but lacking malate synthase apparently use acetyl-CoA for lipid rather than malate synthesis. The alternate path in the *Mycoplasma* species proceeds from PEP to pyruvate via pyruvate kinase, with the gain of one ATP, then from pyruvate to acetyl-CoA by pyruvate dehydrogenase, from acetyl-CoA to malate, then to OAA by malate synthase

and MDH, respectively (Fig. 2, reactions 35, 32, 20 and 14). Therefore, the alternate path to OAA in *Mycoplasma* species yields ATP.

This longer and more energetically favourable path in *Mycoplasma* species may be regulated by cellular levels of CoA-SH, glyoxalate and NAD⁺. However, when CoA-SH, NAD⁺ or glyoxalate are limiting, pyruvate carboxylase activity may be important in the synthesis of OAA, even though there is no gain of ATP. The source of glyoxalate in the Mollicutes is unknown. We have not detected isocitrate lyase activity, nor the glyoxalate cycle in any Mollicutes (unpublished data).

The absence of aspartate aminotransferase activity in *M. gallisepticum* S6 denotes that this metabolic point is disconnected from glycolysis and that there may be no passage of glycolytic carbons via pyruvate and OAA to amino acids, and hence to proteins. Although there are no studies examining the linkage of aspartate to the synthesis of amino acids and proteins in the Mollicutes, there are data showing that aspartate may be required in the synthesis of AMP by Mollicutes, and, therefore, a linkage to nucleic acids appears to be established (Figs 1 and 2) (Pollack *et al.*, 1981; Tryon & Pollack, 1985). However, this hypothesis includes as a corollary the accumulation of fumarate. Although we have detected transitory low levels of fumarate in cells, we know of no role for fumarate in the metabolism of Mollicutes (unpublished data). Stimulation of growth by 2-oxoglutarate (Leece & Morton, 1954; Tourtellotte & Jacobs, 1960) might indicate growth stimulating transaminase activity rather than the presence of the TCA cycle.

As we used crude enzyme preparations and competing reactions cannot be excluded, the rates in Table 1 should be viewed with caution, and used to indicate the presence or absence of activity only.

We detected no difference between the enzyme patterns of the non-fermentative and the fermentative *Mycoplasma* species. The pattern detected for the *Mycoplasma* species is different from that of the *Acholeplasma* species.

Our data have thus categorized a number of metabolic interchange activities at the pyruvate-OAA locus. The interchanges may be controlled by the compounds involved, some of which – e.g. acetyl-CoA, ATP and PEP – are already known to be involved in regulation of the central pathways.

We are indebted to V. V. Tryon for help in preparing cell pellets of *M. genitalium* G-37, and to T. Young and R. Ross for help in preparing *M. hyopneumoniae* J. We wish to express our appreciation to the Graduate School of The Ohio State University and its Dean for their encouragement and generous support.

REFERENCES

- BEAMAN, K. D. & POLLACK, J. D. (1981). Adenylate energy charge in *Acholeplasma laidlawii*. *Journal of Bacteriology* **146**, 1055–1058.
- BEAMAN, K. D. & POLLACK, J. D. (1983). Synthesis of adenylate nucleotides by Mollicutes (mycoplasmas). *Journal of General Microbiology* **129**, 3103–3110.
- BEAMAN, K. D. & POLLACK, J. D. (1984). Enzymatic assimilation of ¹⁴C from NaH¹⁴CO₃ by extracts of *Acholeplasma laidlawii* B-PG9. *Yale Journal of Biology and Medicine* **57**, 897.
- BRIDGER, W. A., RAMALEY, R. F. & BOYER, P. D. (1969). Succinyl coenzyme A synthetase from *Escherichia coli*. *Methods in Enzymology* **13**, 70–74.
- BUCHER, T. & PFLEIDERER, G. (1955). Pyruvate kinase from muscle. *Methods in Enzymology* **1**, 435–440.
- CASTREJON-DIEZ, J., FISHER, T. N. & FISHER, E., II. (1963). Glucose metabolism of two strains of *Mycoplasma laidlawii*. *Journal of Bacteriology* **86**, 627–636.
- COCKS, B. G., BRAKE, F. A., MITCHELL, A. & FINCH, L. R. (1985). Enzymes of intermediary carbohydrate metabolism in *Ureaplasma urealyticum* and *Mycoplasma mycoides* subsp. *mycoides*. *Journal of General Microbiology* **131**, 2128–2135.
- COX, D. L. & BAUGH, C. L. (1976). Carboxylation of phosphoenolpyruvate by extracts of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **129**, 202–206.
- DIXON, G. H. & KORNBERG, H. L. (1962). Malate synthetase from baker's yeast. *Methods in Enzymology* **5**, 633–637.
- ERNST, S. M., BUDDE, R. J. A. & CHOLLET, R. (1986). Partial purification and characterization of pyruvate orthophosphate dikinase from *Rhodospirillum rubrum*. *Journal of Bacteriology* **165**, 483–488.
- FRIIS, N. F. (1975). Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*: a survey. *Nordisk Veterinaermedicin* **27**, 337–339.
- GEORGE, H. A. & SMITBERT, R. M. (1982). Fumarate reduction and product formation by the Reiter strain of *Treponema phagedenis*. *Journal of Bacteriology* **152**, 1049–1059.
- GILL, J. W. (1960). Culture and metabolism of

- Mycoplasma gallisepticum*. *Journal of Bacteriology* **83**, 213–218.
- GOLDBERG, D. M. & ELLIS, G. (1983). Isocitrate dehydrogenase. In *Methods in Enzymatic Analysis*, 3rd edn, vol. 3, pp. 183–188. Edited by H. U. Bergmeyer. New York: Academic Press.
- HOLDSWORTH, E. S. & BRUCK, K. (1977). Enzymes concerned with α -carboxylation in marine phytoplankton. *Archives of Biochemistry and Biophysics* **182**, 87–94.
- HOLLAENDER, R., WOLF, G. & MANNHEIM, W. (1977). Lipoquinones of some bacteria and mycoplasmas, with considerations on their functional significance. *Antonie van Leeuwenhoek* **43**, 177–185.
- HOLMES, B. E. & PIRIE, A. (1932). Growth and metabolism of the bovine pleuropneumonia virus. *British Journal of Experimental Pathology* **13**, 364–370.
- HU, R. Y. & LARDY, H. A. (1969). Malic enzyme. *Methods in Enzymology* **13**, 230–235.
- KLOMKES, M., ALTDORF, R. & OHLENBUSCH, H.-D. (1985). Purification and properties of an FAD-containing NADH oxidase from *Mycoplasma capricolum*. *Hoppe-Seyler's Biological Chemistry* **366**, 963–969.
- KORNBERG, A. (1955). Lactic dehydrogenase of muscle. *Methods in Enzymology* **1**, 441–443.
- LANHAM, S. M., LEMCKE, R. M., SCOTT, C. M. & GRENDON, J. M. (1980). Isoenzymes in two species of *Acholeplasma*. *Journal of General Microbiology* **117**, 19–31.
- LEECE, J. G. & MORTON, H. E. (1954). Metabolic studies on three strains of pleuropneumonia-like organisms isolated from man. *Journal of Bacteriology* **67**, 62–68.
- LIPMAN, R. P., CLYDE, W. A. & DENNY, F. W. (1969). Characterization of virulent, attenuated, and avirulent *Mycoplasma pneumoniae* strains. *Journal of Bacteriology* **100**, 1038–1043.
- LYNN, R. J. (1960). Oxidative metabolism of pleuropneumonia-like organisms. *Annals of the New York Academy of Sciences* **79**, 538–542.
- MCGARRITY, G. J., CONSTANTOPOULOS, G. & BARRANGER, J. A. (1984). Effect of mycoplasma infection on pyruvate dehydrogenase complex activity of normal and pyruvate dehydrogenase complex-deficient fibroblasts. *Experimental Cell Research* **151**, 557–562.
- NEIMARK, H. C. & PICKETT, M. J. (1960). Products of glucose metabolism by pleuropneumonia-like organisms. *Annals of the New York Academy of Sciences* **79**, 531–537.
- POLLACK, J. D. (1975). Localization of reduced nicotinamide adenine dinucleotide oxidase activity in *Acholeplasma* and *Mycoplasma* species. *International Journal of Systematic Bacteriology* **25**, 108–113.
- POLLACK, J. D. (1983). Localization of enzymes in mycoplasma: preparatory steps. *Methods of Mycology* **1**, 327–332.
- POLLACK, J. D. & WILLIAMS, M. V. (1986). PP_i-dependent phosphofructotransferase (phosphofructokinase) activity in the Mollicute (*Mycoplasma*) *Acholeplasma laidlawii*. *Journal of Bacteriology* **165**, 53–60.
- POLLACK, J. D., SOMERSON, N. L. & SENTERFIT, L. B. (1970). Isolation, characterization, and immunogenicity of *Mycoplasma pneumoniae* membranes. *Infection and Immunity* **2**, 326–339.
- POLLACK, J. D., MEROLA, A. J., PLATZ, J. & BOOTH, R. L., II. (1981). Respiration-associated components of Mollicutes. *Journal of Bacteriology* **146**, 907–913.
- RAZIN, S. (1963). Nutritional requirements and metabolism of *Mycoplasma laidlawii*. *Journal of General Microbiology* **30**, 141–154.
- REDDY, T. L. P. & WEBER, M. M. (1986). Solubilization, purification and characterization of succinate dehydrogenase from membranes of *Mycobacterium phlei*. *Journal of Bacteriology* **167**, 1–6.
- REED, J. R. & MUKHERJEE, B. B. (1963). α -Ketoglutarate dehydrogenase from *E. coli*. *Methods in Enzymology* **13**, 55–61.
- REINARDS, R., KUBICKI, J. & OHLENBUSCH, H. D. (1981). Purification and characterization of NADH oxidase from membranes of *Acholeplasma laidlawii*, a copper-containing iron-sulfur flavoprotein. *European Journal of Biochemistry* **120**, 329–337.
- REJ, R. & HORDER, J. (1983). Aspartate aminotransferase (glutamate-oxaloacetate transaminase). In *Methods in Enzymatic Analysis*, 3rd edn, vol. 3, pp. 416–425. Edited by H. U. Bergmeyer. New York: Academic Press.
- RODWELL, A. W. (1960). Nutrition and metabolism of *Mycoplasma mycoides* subsp. *mycoides*. *Annals of the New York Academy of Sciences* **79**, 499–507.
- RODWELL, A. W. & RODWELL, E. S. (1954). The breakdown of carbohydrates by *Asterococcus mycoides*, the organisms of bovine pleuropneumonia. *Australian Journal of Biological Science* **7**, 18–30.
- SALIH, M. M., SIMONSEN, V. & ERNØ, H. (1983). Electrophoretic analysis of isoenzymes of *Acholeplasma* species. *International Journal of Systematic Bacteriology* **33**, 16–172.
- SLAVIK, N. F. & SWITZER, W. P. (1972). Development of a microtitration complement fixation test for the diagnosis of *Mycoplasma suipneumoniae*. *Iowa State Journal for Research* **47**, 117–128.
- SMITH, P. E. (1971). *The Biology of Mycoplasmas*. New York: Academic Press.
- SMITH, T. E. (1968). *Escherichia coli* phosphoenolpyruvate carboxylase: competitive regulation by acetyl coenzyme A and aspartate. *Archives of Biochemistry and Biophysics* **128**, 611–622.
- SRERE, P. A. (1969). Citrate synthase. *Methods in Enzymology* **13**, 3–11.
- STITT, M. (1983a). Citrate synthase (condensing enzyme). In *Methods in Enzymatic Analysis*, 3rd edn, vol. 4, pp. 353–358. Edited by H. U. Bergmeyer. New York: Academic Press.
- STITT, M. (1983b). Fumarase. In *Methods in Enzymatic Analysis*, 3rd edn, vol. 3, pp. 359–362. Edited by H. U. Bergmeyer. New York: Academic Press.
- TOURTELLOTT, M. E. & JACOBS, R. E. (1960). Physiological and serologic comparisons of PPLO from various sources. *Annals of the New York Academy of Sciences* **79**, 521–530.
- TRYON, V. V. & POLLACK, J. D. (1984). Purine metabolism in *Acholeplasma laidlawii* B: novel PP_i-dependent nucleoside kinase activity. *Journal of Bacteriology* **159**, 265–270.
- TRYON, V. V. & POLLACK, J. D. (1985). Distinctions in Mollicutes purine metabolism: pyrophosphate-dependent nucleoside kinase and dependence on

- guanylate salvage. *International Journal of Systematic Bacteriology* **35**, 497–501.
- VANDEMARK, P. J. & SMITH, P. F. (1964*a*). Respiratory pathways in the mycoplasma. II. Pathway of electron transport during oxidation of reduced nicotinamide adenine dinucleotide by *Mycoplasma hominis*. *Journal of Bacteriology* **88**, 12–129.
- VANDEMARK, P. J. & SMITH, P. F. (1964*b*). Evidence for a tricarboxylic acid cycle in *Mycoplasma hominis*. *Journal of Bacteriology* **88**, 373–377.
- VANDEMARK, P. J. & SMITH, P. F. (1965). Nature of butyrate oxidation by *Mycoplasma hominis*. *Journal of Bacteriology* **88**, 1602–1607.
- WILLIAMS, V. R. & LARTIGUE, D. J. (1969). Aspartase. *Methods in Enzymology* **13**, 354–361.
- WOOD, H. G., O'BRIEN, W. E. & MICHAELS, G. (1977). Properties of carboxytransphosphorylase; pyruvate dikinase; pyrophosphate phosphofructokinase and pyrophosphateacetate kinase and their roles in the metabolism of inorganic phosphate. *Advanced Enzymology and Related Areas of Molecular Biology* **45**, 85–155.
- YOSHIDA, A. (1969). L-Malate dehydrogenase from *Bacillus subtilis*. *Methods in Enzymology* **13**, 141–145.