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Activities of Oxidative Enzymes in Mycoplasmas

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The activities of several oxidoreductases were measured in three fermentative and two nonfermentative *Mycoplasma* species that were grown under aerobic or anaerobic conditions. *Acholeplasma laidlawii* MG, *Mycoplasma hyorhinis* GDL, and *Mycoplasma pneumoniae* FH had very high apparent activities of pyruvate dehydrogenase and pyruvate dehydrogenase complex compared with the activities of mammalian fibroblasts or human platelet-enriched preparations, while *Mycoplasma salivarium* VV and *Mycoplasma arthritidis* 07 had very low apparent activities of these two enzymes. Strictly anaerobic growth diminished both enzymatic activities. The activity of α-ketoglutarate dehydrogenase complex was minimal in all five mycoplasmas that were grown under aerobic conditions, anaerobic conditions, or both. All the mycoplasmas that were examined exhibited lactate dehydrogenase and NADH-dichlorophenol indophenol oxidoreductase activities. The properties of mycoplasmal pyruvate dehydrogenase complex suggest that it differs from the mammalian enzyme.

The energy-yielding pathways of mycoplasmas are poorly understood (11). It is known that the fermentative mycoplasmas, when grown aerobically, oxidize glucose to acetate and carbon dioxide (13), but information about the metabolism of pyruvate is scanty. Regarding the nonfermentative mycoplasmas, it has been reported that a tricarboxylic acid cycle may be functioning in *Mycoplasma arthritidis* (18). In this study the activities of pyruvate dehydrogenase complex (PDHC), its first catalytic component pyruvate dehydrogenase (PDH; EC 1.2.4.1), α-ketoglutarate dehydrogenase complex (α-KGDHC; EC 1.2.4.2), lactate dehydrogenase (LDH; EC 1.1.1.27), and NADH-dichlorophenol indophenol (DCPIP) oxidoreductase were measured in homogenates of the fermentative mycoplasmas *Mycoplasma hyorhinis* GDL ATCC 23839, *Acholeplasma laidlawii* MG ATCC 29804, and *Mycoplasma pneumoniae* FH (ATCC 15531) and in homogenates of the nonfermentative *Mycoplasma salivarium* VV ATCC 29803 and *Mycoplasma arthritidis* 07 grown under both anaerobic and aerobic conditions. The results obtained show that *M. hyorhinis* GDL, *A. laidlawii* MG, and *M. pneumoniae* FH have very high apparent activities of PDHC and PDH, while *M. salivarium* VV and *M. arthritidis* have very low activities of these enzymes. The α-KGDHC activity of all five mycoplasma species, when grown under various conditions, was negligible. The properties of the mycoplasmal PDHC suggest that it differs from the PDHC of human fibroblasts.

MATERIAL AND METHODS

Mycoplasmas and growth conditions. *M. hyorhinis* GDL, *M. pneumoniae* FH, *M. salivarium* VV, and *A. laidlawii* MG were obtained from the American Type Culture Collection, Rockville, Md. *M. arthritidis* 07 was obtained from J. G. Tully, National Institutes of Health, Frederick, Md. This organism was originally identified as the 07 strain of *M. hominis*. It is now recognized that *M. hominis* 07 is actually *M. arthritidis* 07 (3). The identity of the organisms was confirmed before each experiment by growth inhibition tests (5). Aerobic, anaerobic, and strictly anaerobic growth started with inocula that were grown anaerobically, aerobically, and anaerobically, respectively. Mycoplasmas, with the exception of *M. pneumoniae* FH, were grown in a medium of mycoplasma broth base (75%; BBL Microbiology Systems, Cockeysville, Md.), horse serum (20%), and yeast extract (5%) and supplemented with 0.1% glucose–0.1% arginine–0.002% phenol red. This medium was modified to 70% broth base, 20% horse serum, and 10% yeast extract for *M. pneumoniae* FH because this yielded better growth. A total of 30 ml of appropriate late-log-phase broth cultures was inoculated into 270 ml of complete medium and incubated at 37°C. Anaerobic conditions were obtained by using GasPaks (BBL). For aerobic incubation, 10 flasks of 30 ml each were used to increase the surface area to volume ratio. Mycoplasma cultures were harvested in late log phase and centrifuged at 15,000 × *g* for 30 min in a rotor (model 30; Beckman Instruments, Inc., Fullerton, Calif.). The pellet was washed in phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 [pH 7.2]) and was suspended and recentrifuged three times.

Fibroblasts and platelet-enriched fractions. Human diploid fibroblast IMR-90 cultures were obtained from the Aging Cell Repository, which is housed at the Coriell Institute for Medical Research. Cell cultures were at population doubling level 30 at the beginning of these studies. Cell cultures were assayed for mycoplasmas by agar and broth inoculation, by DNA staining, and with 6-methylpurine deoxyriboside (9). Cell cultures were maintained in McCoy medium supplemented with 20% fetal bovine serum. Human platelet-enriched fractions were obtained by the procedure described by Blass et al. (2). These preparations consisted mainly of leukocytes.

Preparation of homogenates. The harvested cells (mycoplasmas, fibroblasts, platelets) were suspended in 0.2 to 1.0 ml of a mixture of 40 ml of glycerol with 60 ml of a Tris hydrochloride buffer (pH 7.4) (2). This suspension was homogenized immediately or could be stored at −20 to −40°C for several months. For the preparation of cell homogenates, the cell suspensions were diluted to about 1 mg of protein (or less) per ml with Tris hydrochloride buffer (pH 7.4) containing 1 mM EDTA and 1 mM 2-mercaptoeth-

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TABLE 1. Activities of oxidoreductases in mycoplasmas

Mycoplasma	Type	Mode of growth	Activities of: PDHC[a]	PDH (E_1)[a]	α-KGDHC[a]	LDH[b]	NADH-DCPIP oxidoreductase (diaphorase)[c]
A. laidlawii MG	Fermentative	Anaerobic	2,200	2,156	21	30	31
A. laidlawii MG	Fermentative	Strictly anaerobic	1,710	2,352	0	113	51
A. laidlawii MG	Fermentative	Aerobic	1,200	2,334	11	67	23
M. hyorhinis GDL	Fermentative	Anaerobic	1,050	848	21	803	53
M. hyorhinis GDL	Fermentative	Strictly anaerobic	58	79	10	133	22
M. hyorhinis GDL	Fermentative	Aerobic	7,350	4,242	37	1,210	193
M. pneumoniae FH	Fermentative	Anaerobic	6,812	15,345	74	640	182
M. pneumoniae FH	Fermentative	Strictly anaerobic	2,492	4,758	57	202	162
M. pneumoniae FH	Fermentative	Aerobic	5,308	9,606	65	200	105
M. salivarium VV	Nonfermentative	Anaerobic	100	58	40	48	106
M. salivarium VV	Nonfermentative	Strictly anaerobic	51	44	57	68	97
M. salivarium VV	Nonfermentative	Aerobic	138	31	22	105	65
M. arthritidis 07	Nonfermentative	Strictly anaerobic	49	12	0.5		
Fibroblasts (IMR-90)			565	78	668	3,350 (2,500–4,200)	253
Platelet-enriched preparations (human)			284	5	700	1,020 (920–1,460)	43

[a] Activity is in picomoles per minute per milligram of protein.
[b] Activity is in nanomoles per minute per milligram of protein.
[c] Activity is micromoles of DCPIP reduced per minute per milligram of protein.

anol (2), subjected to three cycles of freeze-thawing, and further dispersed by a few passes in an all-glass hand homogenizer. The methods used for the preparation of homogenates and enzyme assays were optimal for mammalian tissues. It was assumed that osmotic lysis after preloading with glycerol, followed by freeze-thawing, adequately breaks the mycoplasmas. Protein content was determined by the method described by Lowry et al. (8) by using bovine crystalline serum albumin as a standard.

Enzyme assays. The enzymes PDHC and α-KGDHC were assayed by a radiochemical method (2) by using [1-^{14}C]pyruvic acid, sodium salt, and [1-^{14}C]-α-ketoglutaric acid, sodium salt, respectively, as substrates. Mycoplasma homogenates containing 5 to 100 μg of protein were placed in disposable test tubes (12 by 75 mm) in an ice bath and were brought to 200 μl with 0.01 M Tris (pH 7.4) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. A total of 10 μl of a solution containing coenzyme A (CoA), NAD^+, and thymine PP; (each 2 mM) in Tris buffer were added; and the contents were mixed. Then, 10 μl of the respective radioactive substrate (20 nmol) was added, and the contents were mixed thoroughly. A control enzyme preparation was inactivated with 10 μl of 50% trichloroacetic acid. The tubes were stoppered with corks, from which were suspended plastic wells (cellulose propionate tubes; 4.8 by 19.9 mm; catalog no. 341288; Beckman). The wells contained paper wicks that were impregnated with 50 μl of 2.5 M NaOH to absorb the evolved CO_2. Reactions were conducted at 37°C for 30 min. At the end of the incubation period, the tubes were placed in an ice bath, and the reaction was stopped by the addition of 10 μl of 50% trichloroacetic acid. The tubes were stoppered again. Complete CO_2 collection was achieved by shaking the tubes for 2 h at 37°C. The radioactivity was assessed by counting the contents of the wells in 10 ml of Ultrafluor (National Diagnostics, Somerville, N.J.), by standard techniques.

The activity of PDH was determined either by the method described by Reed and Willms (12), which was scaled down to a total volume of 140 μl, or by a recently described modification of the original method (7). Sodium [1-^{14}C]pyruvate was used as the substrate in both methods. The concentration of the substrate was 0.1 mM in the first method and 0.2 mM in the second method. The activity of LDH was measured at 25°C by determining the decrease in A_{340} resulting from the oxidation of NADH in the presence of 1 mM sodium pyruvate (17). Diaphorase (NADH-DCPIP oxidoreductase) was measured by the procedure described by Sakurai et al. (15). Arginine deiminase was assayed as described by Schimke et al. (16).

Preparation of mycoplasmal fractions. Homogenates that were prepared as described above were centrifuged at 15,000 × *g* for 15 min; all homogenates provided a pellet and a supernatant designated 15-KP and 15-KS, respectively. Fractions of 15-KS were centrifuged in an ultracentrifuge (Airfuge; Beckman) by using the A-100 fixed angle rotor at a 160,000 × *g* maximum for 2 h at 23°C. The supernatant was removed and designated 160-KS. Any material deposited at the bottom of the tubes ws carefully recovered in Tris hydrochloride–2-mercaptoethanol–EDTA buffer (pH 7.4) with the aid of a glass rod and was designated 160-KP.

Chemicals. [1-^{14}C]pyruvic acid, (sodium salt; 5 to 20 mCi/mmol) and [1-^{14}C]-α-ketoglutaric acid (sodium salt; 40 to 60 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). Nonradioactive sodium pyruvate and sodium α-ketoglutarate, CoA, TPP, NAD^+, NADH, nicotinamide, lipoamide, dichlorophenol indophenol DCPIC, and *N*-Tris(hydroxymethyl)methyl glycine (Tricine) were obtained from Sigma Chemical Co., St. Louis, Mo.).

RESULTS

In Table 1 are shown the species of mycoplasma that were examined; their type and mode of growth; and the activities of PDHC, PDH, α-KGDHC, LDH, and NADH-DCPIP oxidoreductase under the various conditions of growth. Homogenates of *A. laidlawii*, *M. hyorhinis*, and *M. pneumoniae* that were grown aerobically or anaerobically had at least 2 times higher PDHC and PDH specific activities than homogenates of fibroblasts IMR-90 and human platelet-enriched preparations. Specifically, the PDHC activity of *A. laidlawii*, *M. hyorhinis*, and *M. pneumoniae* was 2 to 5, 2 to 13, and 10 to 12 times higher, respectively, than the PDHC activity of fibroblasts. In general, the activity of PDH was higher than that of PDHC. The PDH activity of *M. pneumo-*

TABLE 2. Cofactor requirements of PDHC from various sources

Reaction mixture[a]	PDHC activity (% of complete activity) of[b]:			
	Aerobically	Anaerobically	Fibroblasts	Platelet-enriched preparations
Complete	100.0 (6,650)[c]	100.0 (905)	100.0 (521)	100.0 (219)
−TPP	10.0	7.2	12.6	9.1
−NAD⁺	44.0	44.5	17.0	16.2
−CoA	59.5	35.6	9.9	5.8
−NAD⁺, −TPP	7.7	8.9	8.2	7.0
−CoA, −TPP	9.4	9.6	4.8	4.5
−NAD⁺, −CoA	38.4	40.5	10.3	5.2
−TPP, −NAD⁺, −CoA	7.4	10.1	5.4	1.7

[a] The concentrations of TPP, NAD^+, and CoA were each 90 μM in the complete reaction mixture.
[b] PHDC activity in picomoles per minute per milligram of protein, before adjustment to 100 is given in parentheses. Cell homogenates were prepared and their PDHC activity was determined as described in the text. The protein concentration of the incubation mixture was 100 μg/ml for *M. hyorhinis* GDL grown aerobically, 225 μg/ml for *M. hyorhinis* GDL grown anaerobically and for fibroblasts, and 450 μg/ml for platelet-enriched preparations.

niae grown anaerobically was about 200 times higher than the respective activity of fibroblasts. Strictly anaerobic growth of *M. hyorhinis* and *M. pneumoniae* resulted in diminished PDHC and PDH activities. In contrast to the fermenters, the PDHC and PDH activities of the nonfermentative organisms *M. salivarium* and *M. arthritidis* were very low.

The presence of PDHC in *M. salivarium* and *M. arthritidis* was ascertained after fractionation. After centrifugation for 15 min at 15,000 × *g*, essentially all PDHC activity of the fibroblast and platelet homogenates was pelleted, while approximately 30 to 50% of that of the mycoplasmas remained in the supernatant obtained after centrifugation at 15,000 × *g*. The specific activity of the 160-KP fraction, in picomoles per minute per milligram of protein, was 487 ± 340 (standard deviation) for *M. salivarium* and 172 for *M. arthritidis* compared with 148 ± 30 (standard deviation) and 49 in their whole homogenates, respectively.

The α-KGDHC activity of mycoplasmas ranged from 0 to 74 pmol/min per mg of protein (Table 1). However, the rate of the enzymatic reaction was not proportional to the amount of homogenate. Also, the substrate [1-^{14}C]-α-ketoglutarate conceivably contained up to 0.1% pyruvate (data obtained from the manufacturer). Therefore, the recovered $^{14}CO_2$ may be derived from [1-^{14}C]pyruvate. All mycoplasma species that were examined had LDH and NADH-DCPIP oxidoreductase activities. Arginine deiminase activity, in micromoles per minute per milligram of protein, was 0.65 in *M. arthritidis*, which was strictly anaerobic; 2.43, 2.03, and 1.76 in *M. salivarium*, which were anaerobic, strictly anaerobic, and aerobic, respectively; and it was not detectable in *A. laidlawii* and *M. hyorhinis* grown under various conditions.

TABLE 3. Effect of sodium arsenite on the PDHC activity of mycoplasmas, fibroblasts, and human platelet-enriched preparations

Sodium arsenite concn (mM)	% Inhibition of PHDC activity by:				
	A. laidlawii MG	*M. hyorhinis* GDL	*M. pneumoniae* FH	Fibroblasts	Platelet-enriched preparations
0.01	23.6	14.8	9.5	27.3	40.5
0.02	26.6	15.0	14.3	42.2	42.2
0.05	47.3	32.8		58.3	70.9
0.10	54.3	41.7	16.0	66.6	77.2
1.00	59.2	58.0	43.0	85.0	88.0

The omission of TPP from the reaction mixture resulted in about a 90% loss of the PDHC activity in both mycoplasmal and mammalian preparation (Table 2). Omission of NAD^+, CoA, or both also resulted in about 90% loss of PDHC activity in the mammalian cells. However, only about one-half of the activity was lost in the mycoplasmas. In general, mycoplasmal PDHC activity was less sensitive to sodium arsenite (Table 3). The Michaelis-Menten constant of PDHC ranged from 14 μM for platelet-enriched preparations to 74 μM for aerobically grown *A. laidlawii*, and the V_{max} ranged from 368 pmol/min in platelets to 10,931 pmol/min in *M. hyorhinis* (Table 4). Heat inactivation at 50°C of PDHC in homogenates from various sources is shown in Fig. 1. The PDHC of *A. laidlawaii*, when it was grown either aerobically or anaerobically, and of *M. hyorhinis* and *M. pneumoniae*, when they were grown anaerobically, was more resistant to heat inactivation than the mammalian PDHC and that of *M. hyorhinis* and *M. pneumoniae* when they were grown aerobically.

DISCUSSION

The results of this study indicate that the fermentative mycoplasmas *A. laidlawii* and *M. pneumoniae* have very high apparent PDHC and PDH activities, suggesting that the oxidation of pyruvate may be another major pathway via which glucose provides energy in these microorganisms. In agreement with results presented in a previous report (1), these mycoplasmas do not have detectable arginine deiminase activity; thus, they probably do not obtain energy from arginine by the arginine dihydrolase pathway. In contrast, the nonfermentative mycoplasmas *M. salivarium* and *M. arthritidis* have low PDH and PDHC activities. However, in accordance with resulted presented in a previous report (1), they have very high arginine deiminase activity. Oxidation of pyruvate by *M. mycoides* was reported in the 1950s (14). High apparent PDHC and PDH activities com-

TABLE 4. Kinetic data of mycoplasma, fibroblast, and platelet PDHC

Source	K_m (mM)[a]	V_{max} (pmol/min)[a]
A. laidlawii MG, aerobic	0.074	2,616
A. laidlawii MG, anaerobic	0.042	2,790
M. hyorhinis GDL, aerobic	0.057	10,931
Fibroblast (IMR-90)	0.042	690
Platelet-enriched preparations	0.014	368

[a] Values are averages of two determinations.

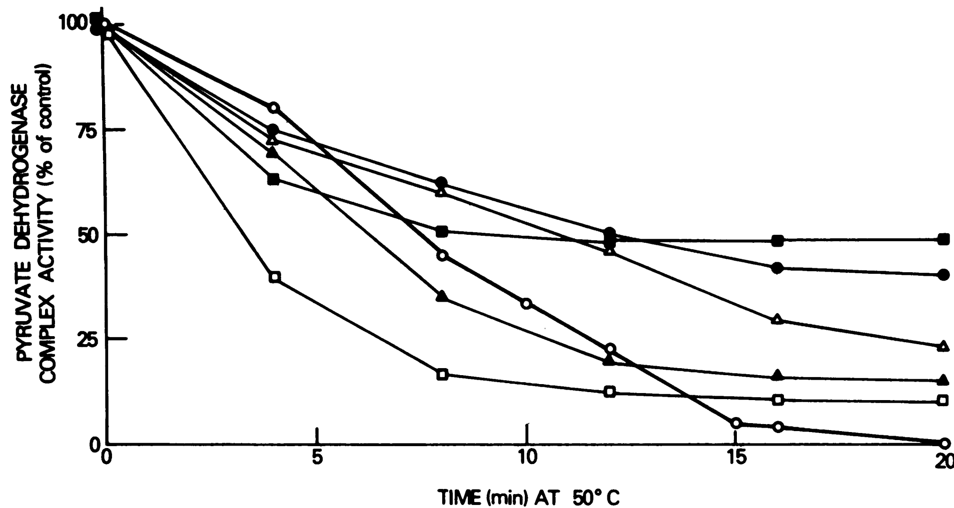

FIG. 1. Heat inactivation of PDHC in homogenates of mycoplasmas, fibroblasts, and platelet-enriched preparations at 50°C. Symbols: ○, Fibroblast and platelet-enriched preparations (protein concentration, 2 mg/ml); ●, *A. laidlawii* JS, strictly anaerobic and aerobic (protein concentration, 0.5 mg/ml); ■, *M. hyorhinis* GDL, anaerobic (protein concentration, 1 mg/ml); □, *M. hyorhinis* GDL, aerobic (protein concentration, 0.2 and 1.0 mg/ml); △, *M. pnemoniae* FH, anaerobic (protein concentration, 0.5 mg/ml); ▲, *M. pneumoniae* FH, aerobic (protein concentration, 0.5 mg/ml). All samples in homogenization mixture (pH 7.4; see text) were incubated at 50°C for 0 to 20 min. The samples were chilled rapidly to 0°C and further diluted and assayed as described in the text. The values are expressed as a percentage of the activity of an unheated sample.

pared with those of human fibroblasts were reported in *M. pneumoniae* by Clark et al. (4), who also reported a fourfold higher activity of α-KGDHC. In this study the activity of α-KGDHC, if present at all, was minimal in all species of mycoplasma that were examined, including *M. pneumoniae*. The activity of PDHC and PDH diminished when the mycoplasmas were grown under strictly anaerobic conditions, suggesting that these mycoplasmal enzymes are inducible or are subject to other controls.

By determination of PDH (E1) activity in mammalian cell homogenates by the pyruvate and TPP-dependent reduction of ferricyanide, only a fraction of the total activity is measured (4). However, in mycoplasmas PDH activity measured by the same method was equal or even higher than the respective PDHC activity. This observation may suggest the presence of uncomplexed E1 in mycoplasmas. Also, the difference in sedimentation rate between mammalian and mycoplasmal PDHC may suggest the production of finer membrane pieces by cell disruption, looser organization of the mycoplasmal PDHC, or both. The higher PDHC activity of fraction 160-KP, compared with that of whole homogenates (Table 1), suggests that fraction 160-KP consists of membrane fragments and that mycoplasmal PDHC is localized in the membrane.

The difference in NAD^+ and CoA requirements that were found between mycoplasmal and mammalian cell homogenates may only be apparent, as there may be sufficient quantities of endogenous NAD^+ and CoA to satisfy the cofactor requirement. However, the general insensitivity of the mycoplasmal PDHC to inhibition by sodium arsenite, the greater stability to heat inactivation at 50°C, the different distribution of PDHC activity after sedimentation of the disrupted cells, and in particular, the high PDH values obtained with ferricyanide as the electron acceptor suggest that the mycoplasmal PDHC differs from the mammalian PDHC. Definitive results await the isolation and characterization of the mycoplasmal enzyme.

It has been suggested (18) that a functioning tricarboxylic acid cycle is present in the nonfermentative *M. arthritidis* (*M. hominis*) 07. We had some difficulty in the aerobic growth of this organism; but the minimal α-KGDHC activity of all mycoplasmas examined, which were grown either aerobically or anaerobically, could be compared in particular with the very high apparent PDHC activity of the fermentative species and suggests the absence of an operating tricarboxylic acid cycle. Perhaps ATP could be generated in several fermentative and nonfermentative mycoplasmas from acetyl CoA by the combined action of phosphate acetyl transferase and acetate kinase, as has been suggested by Kahane et al. (6). Phosphate acetyl transferase activity has been reported in *A. laidlawii* and *M. hominis* (6); and acetate kinase activity has been reported, among others, in *A. laidlawii*, *M. hominis*, *M. pneumoniae*, *M. salivarium*, and *Mycoplasma gallisepticum* (10). Pyruvate may also be metabolized by other pathways.

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