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Analysis of Multiple Isoenzyme Expression Among Twenty-Two Species of *Mycoplasma* and *Acholeplasma*

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Crude extracts of triple-cloned, purified cultures of 22 species of *Mycoplasma* and *Acholeplasma* were examined for expression of 21 isozyme systems routinely used to type mammalian cells. Nine previously described enzymes (purine nucleoside phosphorylase, adenylate kinase, dipeptidase, esterase, glyceraldehyde-3-phosphate dehydrogenase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and superoxide dismutase) and three enzymes not previously reported in mycoplasmas (triose phosphate isomerase, inorganic pyrophosphatase, and acid phosphatase) were detected in some or all of the species examined. These findings provide new information on the enzymatic expressions of these organisms. Three of the isozyme systems (superoxide dismutase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase) were present in *Acholeplasma* species but not in any *Mycoplasma* species. The characteristic pattern of electrophoretic mobility of the 12 isozyme systems also provides a useful biochemical property for identification, characterization, and classification of these mycoplasmas. Mycoplasma isozyme expression for seven of the enzymes were readily detected in various infected-cell culture lines by using either cell extracts or concentrated cell culture fluids. Mycoplasma-specific enzymes found in infected-cell extracts had the same electrophoretic mobility patterns as enzymes obtained from broth-grown mycoplasmas of the same species. Expression of homologous mammalian enzymes was not detectably altered by infection with mycoplasmas.

Over 70 different species of mycoplasmas have been described (7) since their initial isolation some 80 years ago (26). Mycoplasmas are the smallest procaryotic organisms capable of in vitro cultivation; the smallest units are 200 to 300 nm in diameter, with genome sizes ranging from 0.5×10^9 to 1.0×10^9 daltons. Unlike bacteria, they lack a cell wall and often parasitize mammalian cells by attaching to the plasma membrane. They are commonly isolated from humans and animals and are the causative agents of pleuropneumonia, urogenital infections, and arthritis in a number of avian and mammalian hosts (39).

Mycoplasmas can produce covert infection of cell cultures or they can cause severe cytopathic effects, depending on the infecting species and cell line used (2-4). Morphological and metabolic cellular functions can also be altered as a result of infection (2-5). Commonly observed changes in infected cells (2-5, 12, 38, 39) include (i) alteration (or transformation) of cell morphology and growth pattern, (ii) increase in chromosome aberrations, (iii) modification of nucleic acid metabolism, (iv) rapid depletion of required nutrients in culture medium (e.g., arginine, leading to cell starvation), (v) interference in cell membrane phenomena such as membrane receptor-mediated lymphocyte transformation and ciliary function, (vi) alteration of proliferation of certain mammalian viruses, and (vii) the production and release of mycoplasma-specific enzymes into the cellular and extracellular environment contributing to alterations of metabolite levels in these compartments.

The results of a biochemical survey of 22 mycoplasmas are described for expression and identification of 21 soluble enzyme systems commonly used for specific genetic characterization of cultured mammalian cells (18, 27, 30). The same enzymes were examined in cell culture lysates and in medium fluid exudates of three cell lines infected with a number of mycoplasmas. The electrophoretic patterns of these enzymes were investigated (i) to identify biochemical genetic characteristics of these organisms, (ii) for possible use in identification and classification of established mycoplasmas and new

isolates, (iii) to evaluate the use of mycoplasma-specific isozyme systems as specific markers for detection of cell culture contamination, and (iv) to examine the effect of mycoplasma infection on the expression of mammalian-encoded enzymes. The results obtained indicate that (i) isozyme systems are widely expressed in broth-grown cultures of mycoplasmas, (ii) the pattern of electrophoretic mobility and the amount of enzyme expressed are dependent on the given species examined, and (iii) mycoplasma-specific isozyme systems can often but not always be detected in infected mammalian cell cultures.

MATERIALS AND METHODS

Mycoplasmas and growth conditions. For the sources of the 17 *Mycoplasma* and 5 *Acholeplasma* species and the strains used, see Tables 2 and 3. All mycoplasmas used were triple filter-cloned and purified, and the species designation of each culture was verified by the epiimmunofluorescence procedure (6). Each mycoplasma used was grown in 1- to 2-liter volumes of broth medium containing mycoplasma broth base (BBL Microbiology Systems, Cockeysville, Md.), 10% yeast extract (Microbiological Associates, Inc., Bethesda, Md.), 0.5% glucose for fermenting or 0.1% arginine-hydrochloride for nonfermenting, arginine-utilizing species, and 100 U of penicillin G per ml. Medium was supplemented with either 10 to 20% horse serum (Microbiological Associates, Inc., Bethesda, Md.) for the *Mycoplasma* species or 2 to 5% PPLO bovine serum fraction (Difco Laboratories, Detroit, Mich.) for the *Acholeplasma* species (6). Broth cultures were incubated at 35 ± 1°C for 2 to 5 days, depending on the species, and the cells were sedimented by centrifugation at 20,200 × *g* for 60 min at 4°C. The mycoplasma cell pellet was suspended and washed twice with phosphate-buffered saline, pH 7.4, before use. The mycoplasma titer in each culture was determined by either colony-forming units (CFU) per milliliter, using agar medium, or color-changing units (CCU) per milliliter, using broth medium.

Cell cultures and culture conditions. The following mycoplasma-free cell cultures were obtained from the American Type Culture Collection: African green monkey kidney Vero (CCL81), mouse L-929 (CCL1), human HeLa (CCL2), and HEp-2 (CCL 23), a human HeLa derivative (17, 23). Cells were grown in T75 plastic flasks containing 28 ml of Eagle minimum essential medium or Dulbecco-Vogt medium supplemented with 10% heat-inactivated fetal bovine serum. Antibiotics were not used.

Mycoplasma infection of cell cultures. Six T75 flasks each containing either HEp-2, Vero, or L-929 cell lines were infected with 10^6 to 10^7 CFU or CCU of 1 of 13 *Mycoplasma* species. Cell cultures were observed for signs of maximum infection (as determined by changes in pH, cytopathic effects, etc.) and harvested (usually within 3 to 6 days) for tests. The number of mycoplasmas per milliliter of tissue culture medium fluids was determined by the CFU or CCU (or both) per milliliter. The extent of mycoplasma infection of cell cultures was monitored by a specific immunofluorescence titer, using reference fluorescein-conjugated antiserum (12), and by a nonspecific DNA-binding fluorochrome (bisbenzimidazole) stain (3). The number of mycoplasmas attached per mammalian cell was enumerated by direct counting of attached particles in stained fluorescent preparations.

Preparation of concentrated cell culture fluids. Culture medium fluids of infected-cell (and uninfected control) cultures from nine subconfluent T75 flasks (10 ml) were centrifuged at 250 × *g* at 4°C for 5 min to remove most of the mammalian cells. The supernatant was centrifuged for 2 h at 27,000 × *g* at 4°C to harvest the noncytadsorbing mycoplasmal cells present in medium fluids. The resuspended supernatant was decanted by aspiration, and the pellet was suspended in a total of 0.6 ml (concentration factor, 150-fold) of hypotonic extraction buffer (0.05 M Tris-hydrochloride, pH 7.1, 0.001 M EDTA, 0.2% Triton X-100). The suspension was sonicated for 60 s, using a Heat Systems microprobe sonic disrupter, and centrifuged at 27,000 × *g* for 30 min. The supernatant was frozen and stored at −70°C.

Preparation of cell culture lysates. Cell lysates were prepared as described previously (30). Cell cultures were harvested by scraping monolayers with a Teflon scraper, and the cells were washed by using low-speed centrifugation. Cell culture pellets were suspended in 1 to 2 volumes of hypotonic extraction buffer and sonicated as described above. The crude extract was centrifuged for 1 h at 27,000 × *g* at 4°C, and the supernatant was stored at −70°C until used.

Broth-grown mycoplasma extracts. These were prepared by sonication of the sedimented cell pellets suspended in 1 to 2 volumes of hypotonic extraction buffer as described for the cultured cells. Protein concentrations were determined by the Lowry method (20).

Isozyme electrophoresis and stain development. The vertical starch gel electrophoresis apparatus (Buchler Instruments, Fort Lee, N.J.) in combination with 12% Electrostarch (Electrostarch Co., Madison, Wis.) was used to resolve all of the isozymes examined. Four buffer systems (30) were used as follows: TC buffer was a solution of 0.14 M Tris in 0.043 M citric acid, pH 7.1. The conductivity of TC buffer was adjusted to 3.3 mΩ. Undiluted TC buffer (1× TC) was used in the cathode chamber and buffer diluted 0.8× was used in the anode buffer. The TC gel buffer was diluted 0.07×. TEB buffer was a solution of 0.18 M Tris, 0.004 M EDTA, and 0.1 M boric acid, pH 8.6; conductivity was 1.3 mΩ. Undiluted TEB buffer was used in the cathode, and buffer diluted 0.8× was used in the anode. The TEB gel buffer was diluted 0.1×. For NADP dehydrogenases, 1 ml of NADP (20 mg/ml) was added to the cathode buffer for both TC and TEB buffer systems. TEM buffer was a solution of 0.1 M Tris-hydrochloride, 0.01 M Na_2 EDTA, 0.1 M maleic acid, and 0.01 M $MgCl_2$, pH 7.4. Undiluted TEM buffer was the electrode buffer, and TEM gel buffer was diluted 0.1×. PI buffer was a solution of 0.1 M sodium phosphate, pH 6.5 (PI buffer). The bridge was undiluted PI buffer, and the gel was diluted 0.1×. Electrophoresis was carried out for 16 to 20 h at 130 V measured across the gel with a voltmeter. Starch gels were sliced horizontally by using a piano wire slicer,

and histochemical enzyme stain was applied. The rationale for and the components of the isozyme stains have been published elsewhere (14, 24, 30, 35).

RESULTS

Expression of isozymes in broth-grown mycoplasmas. Each mycoplasma species examined was grown in 1 to 2 liters of broth and produced titers ranging from 10^5 to 10^8 CFU or CCU per ml. The crude extract preparations were subjected to gel electrophoresis and assayed by histochemical staining for 21 isozyme systems readily detected in cultured mammalian cells (Table 1). In most mycoplasmas, appreciable activity was detected with nine of the isozyme systems (Fig. 1 and 2; Table 2). Three enzymes were restricted to *Acholeplasma* species (superoxide dismutase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase). The remaining nine isozyme systems were not detected in any of the species. Of the 12 systems present in mycoplasmas, 7 were also detected in mycoplasma-infected mammalian cell cultures. The distribution of enzyme activities observed in different mycoplasmas is shown in Table 2, and their relative electrophoretic mobilities are shown in Fig. 1 and 2. Five of the enzymes (esterase, adenylate kinase, dipeptidase, nucleoside phosphorylase, and inorganic pyrophosphatase) were present in all or most of the mycoplasmas tested, whereas seven of the positive enzyme systems (glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, glucose phosphate isomerase, acid phosphatase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase) appeared in only certain species tested.

The enzymes observed among the mycoplasmas varied in their activities, their electrophoretic mobilities, and in the number of isozymes resolved. These variations were due, in part, to the amount of growth obtained in broth, the stability of enzymes through extraction, the activity under our assay conditions (which are optimal for eucaryotic enzymes), and a variety of undefined parameters (29) known to affect enzyme activities. The electrophoretic mobilities of mycoplasma enzyme systems shown in Fig. 2 provide a series of characteristic genetic

TABLE 1. *Expression of mycoplasma enzymes from broth-grown and infected-cell cultures*

Enzyme	EC no.	Buffer[a]	Substrate	Presence (+) or absence (−) of mycoplasma enzyme		
				Broth	Tissue culture cells	Concentrated cell fluids
Adenylate kinase	2.7.4.3	TCA	ADP	+	+	+
Dipeptidase	3.4.11.-	TEB	L-Leucyl-L-tyrosine, L-valyl-L-leucine	+	+	+
Esterase	3.1.1.1	TEB	α-Napthyl acetate	+	−	−
Glucose phosphate isomerase	5.3.1.9	TEB	Glucose-1-phosphate	+	+	+
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	TEM	Glyceraldehyde-3-phosphate	+	+	+
Purine nucleoside phosphorylase	2.4.2.1	TEB	Inosine, adenine	+	+	+
Inorganic pyrophosphatase	3.6.1.1	TEM	Sodium pyrophosphate	+	−	−
Superoxide dismutase	1.15.1.1	TEB	MTT formazan	+[b]	−	−
Triose phosphate isomerase	5.3.1.1	TEM	Glyceraldehyde-3-phosphate	+	+	+
Acid phosphatase	3.1.3.2	PI	4-Methyl umbelliferyl phosphate	+	+	+
Glucose-6-phosphate dehydrogenase	1.1.1.49	TEB	Glucose-6-phosphate	+[b]	ND[c]	ND
Phosphogluconate dehydrogenase	1.1.1.44	TEB	6-Phosphogluconate	+[b]	ND	ND
Adenosine deaminase	3.5.4.4		Adenosine	−	−	−
Glutamate-oxaloacetate transaminase	2.6.1.1		Aspartate, α-ketoglutarate	−	−	−
Glutathione reductase	1.6.4.2		Oxidized glutathione	−	−	−
Glyoxalase	4.4.1.5		Methyl glyoxal, glutathione	−	−	−
Hexosaminidase	3.2.1.30		4-Methyl umbelliferyl *N*-acetyl glucosamide	−	−	−
Isocitrate dehydrogenase	1.1.1.42		Isocitrate	−	−	−
Malate dehydrogenase	1.1.1.37		Malate	−	−	−
Malic enzyme	1.1.1.40		Malate	−	−	−
Mannose phosphate isomerase	5.3.1.8		Mannose-6-phosphate	−	−	−

[a] For definitions, see the text. The buffer system that gave optimum resolution was subsequently employed.
[b] Observed exclusively in *Acholeplasma* species.
[c] ND, Not determined.

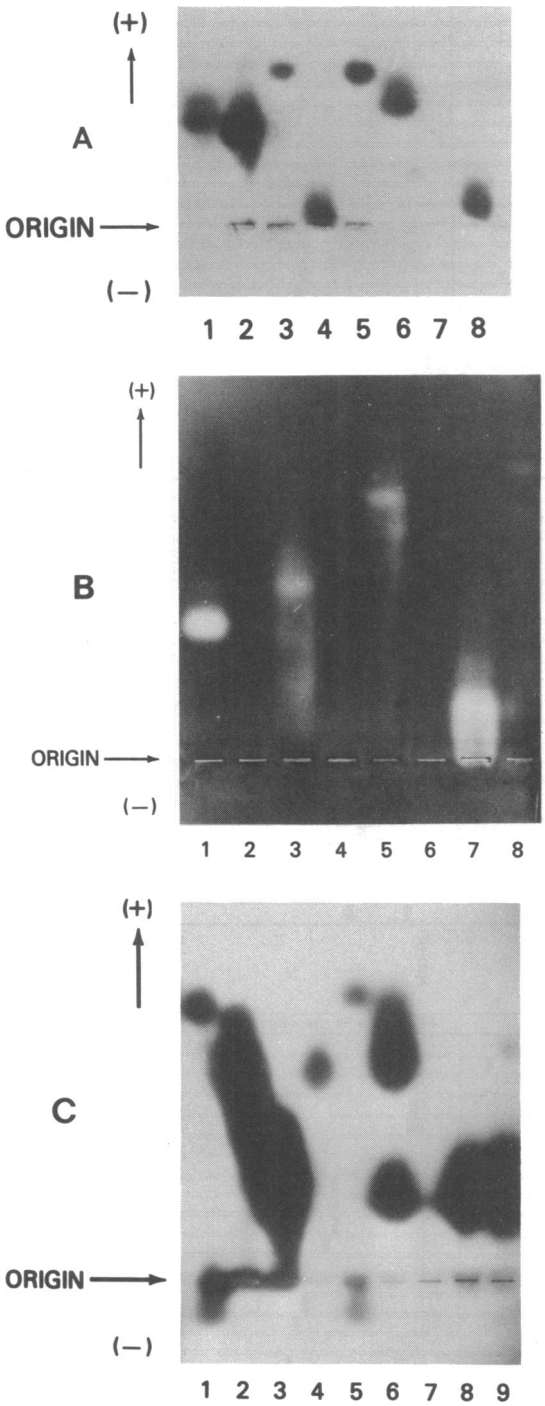

FIG. 1. *Electropherograms of three enzyme strains with extracts of broth-grown mycoplasmas (A and B) and mycoplasma-infected tissue culture cells (c). A. Inorganic pyrophosphatase in extracts of broth-grown mycoplasmas. (1) L cells; (2) M. neurolyticum; (3) A. gallisepticum; (4) M. fermentans; (5) A. granularum; (6) M. anatis; (7) A. laidlawii; (8) M. pneumoniae. B. Superoxide dismutase in extracts of broth-grown mycoplasmas. (1) A. axanthum; (2) M. conjuctivae; (3) A. oculi; (4) M. arginini; (5) A. granularum; (6) M. anatis; (7) A. modicum; (8) HeLa cells. C. Triose phosphate isomerase extracts of broth-grown mycoplasmas and mycoplasma-infected tissue culture cells. (1) M. hyorhinis; (2) A. laidlawii; (3) L cell (uninfected cell) lysate; (4) L cell (A. laidlawii) cell fluid; (5) L cell (M. hyorhinis 1050) cell fluid; (6) HEp-2 (A. laidlawii) cell fluid; (7) HEp-2 (uninfected) cell fluid; (8) HEp-2 (M. hyorhinis 1050) cell lysate; (9) HEp-2 (A. laidlawii 1050) cell lysate. This gel was intentionally overdeveloped to reveal mycoplasma-specific isozymes in infected mammalian cells.*

markers. The electrophoretic resolution of these isozyme systems can be used to identify and classify mycoplasmas in a manner similar to that used for identification of mammalian cells (28, 30).

The enzymes detected in mycoplasmas included three glycolytic enzymes, glucose phosphate isomerase, triose phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase. As expected, mycoplasmas previously classified as glucose fermenters (38) expressed all three enzymes. With two exceptions, the nonfermenting mycoplasmas lacked the three glycolytic enzymes. The exceptions were low levels of triose phosphate isomerase in *M. meleagridis* and the appearance of glucose phosphate isomerase in one strain (1105) of *M. hominis*. Four other strains of *M. hominis* (PG21, H34, 1257, and 760) did not express detectable glucose phosphate isomerase. The basis of enzyme expression in these two exceptional cases is presently under investigation.

Expression of mycoplasma-specific isozymes in infected mammalian cells and cell exudate. Two mammalian fibroblast cell lines (HEp-2 [human] and L cell [murine]) were infected with each of 13 mycoplasmas listed in Table 3, and Vero (monkey) cells were infected with *M. hyorhinis*. The infection was monitored by immunofluorescence, and the cells were harvested just before onset of cytotoxic effects. The cellular lysates (containing cytadsorbing mycoplasmas) and the sedimented cell fluids (containing noncytadsorbing mycoplasmas) were assayed for 10 enzymes detected in broth-grown mycoplasmas (Table 1). Figure 1C presents an example of results of an assay for triose phosphate isomerase in infected cells and supernatant fluids. The results for all enzymes are shown in Table 3.

In general, the expression of mammalian cellular enzymes was not detectably altered by the mycoplasma infection. There were, however, a

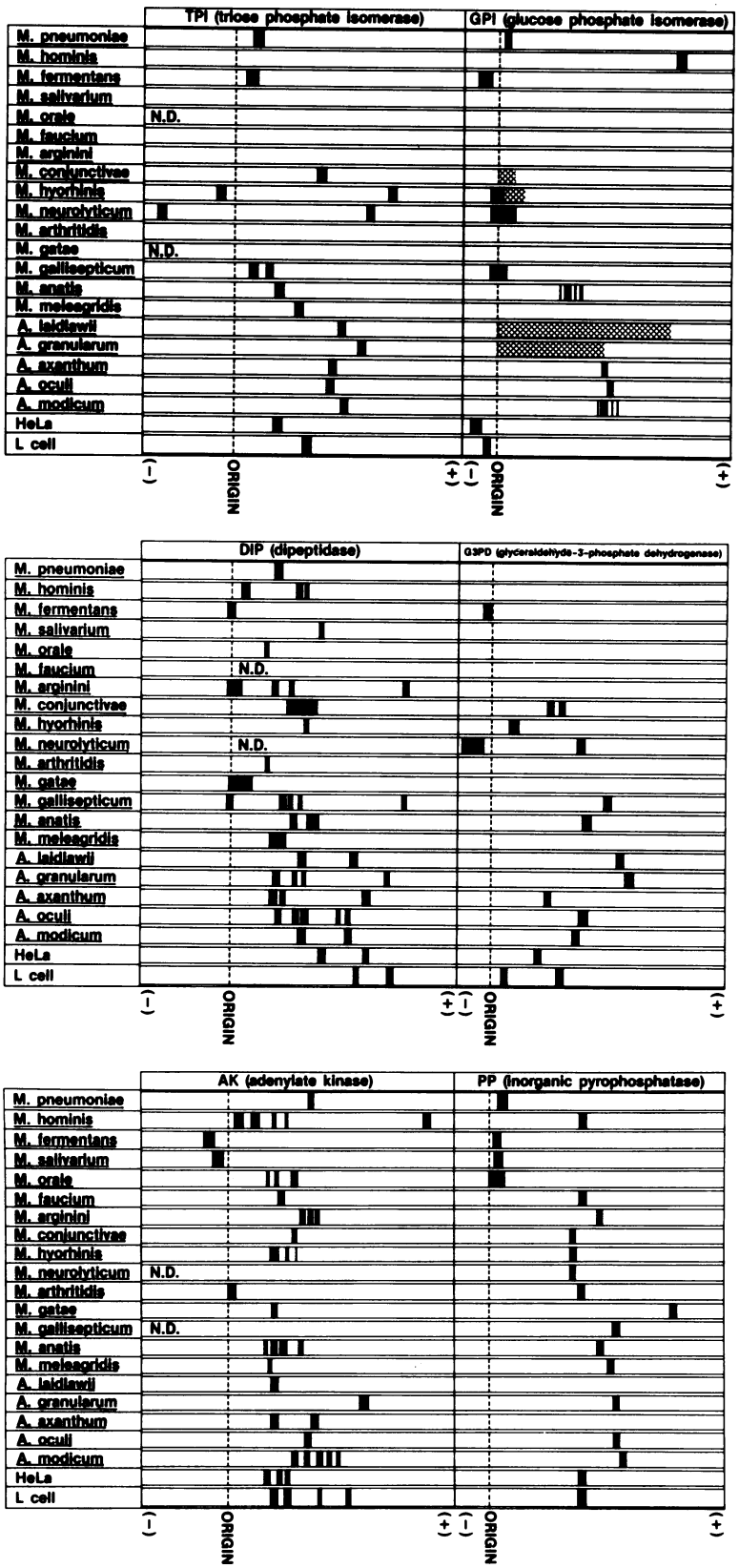

FIG. 2. *Composite diagrammatic electropherogram of 12 isozyme systems detected in 20 broth-grown species of Mycoplasma and Acholeplasma. Species designations refer to the strains listed in Table 2. Mobilities are relative to HeLa and L cells. A blank lane indicates an absence of detected activity in mycoplasma extracts which were positive for other enzymes. ND, Not done.*

number of enzyme stains which revealed additional bands with an electrophoretic mobility identical to that of the same enzyme found in broth-grown extracts of the infecting mycoplasmas (Fig. 1C). These new enzyme bands were considered to be encoded by the mycoplasma genome because their mobilities were constant with the mycoplasmas and independent of the host (broth, human, or murine cell culture). Of the 12 enzymes expressed in broth-grown mycoplasmas, 7 were observed as mycoplasma-specific bands in some, but not all, extracts of cultured cells or in concentrated culture fluid extracts (Table 3). Three of the enzymes (esterase, inorganic pyrophosphatase, and superoxide dismutase) revealed no mycoplasma-specific bands in extracts of infected tissue culture cells. Two enzymes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), which were detected only in an *Acholeplasma* species, were not thoroughly tested in cultured-cell lysates.

Two infections (Vero with *M. hyorhinis* and L cell with *A. laidlawii*) were positive for mycoplasma isozymes in every case except for glyceraldehyde-3-phosphate dehydrogenase in the L cells. These two species produced heavy infections, with 10^{10} mycoplasmas per infected cell culture monolayer in one T75 flask. Most, but not all, of the other infections had fewer detectable mycoplasmas per infected cell culture (10^7 to 10^9). These observations suggest that detection of mycoplasma-specific isozymes in infected cells is dependent upon both the titer and iden-

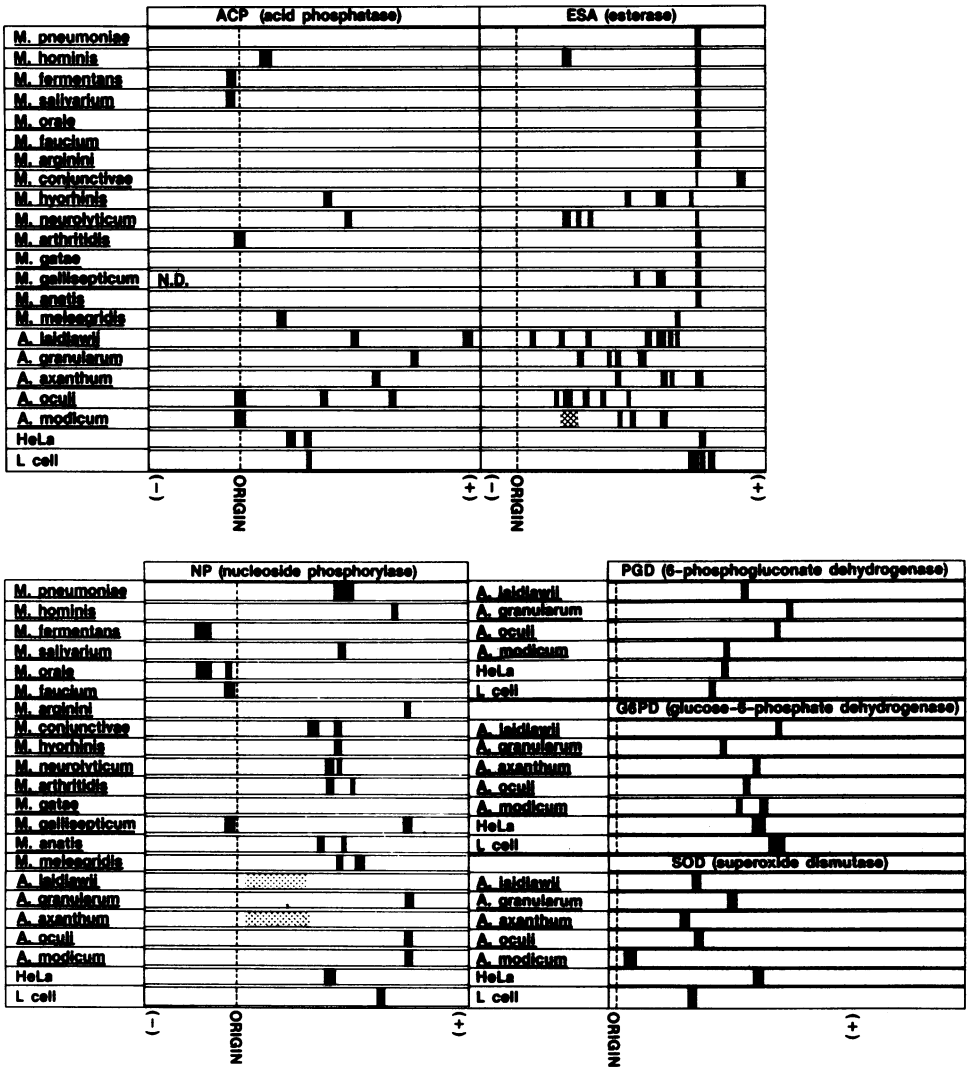

FIG. 2. *Continued*

TABLE 2. *Isozyme expression in broth-grown Mycoplasma and Acholeplasma species*

Species and origin	Protein concn. (mg/ml)[a]	Presence (+) or absence (−) of isozyme system[b]											
		TPI	G3PD	GPI	NP	PP	AK	DIP	ESA	SOD	ACP1	G6PD	PGD
Primate													
M. pneumoniae FH (f)[c] (RRB)[d]	95	+	ND[e]	+	+	+	+	+	+	−	−	−	−
M. hominis PG21 (n) (BB)	172	−	−	−	+	+	+	+	+	−	+	−	−
M. fermentans PG18 (f) (RRB)	11	+	+	+	+	+	+	+	+	−	+	−	−
M. salivarium PG20 (n) (RRB)	39	−	−	−	+	+	+	+	+	−	+	−	−
M. orale CH19299 (n) (RRB)	105	ND	−	−	+	+	+	+	+	−	−	ND	ND
M. faucium DC333 (n) (RRB)	16	−[f]	−	−	+	+	+	ND	+	+[g]	−	−	−
Ovine or caprine													
M. arginini G230 (n) (RRB)	150	−	−	−	+	+	+	+	+	−	−	−	−
M. conjunctivae HRC581 (f) (BB)	ND	+	+	+	+	+	+	+	+	−	−	ND	ND
Swine													
M. hyorhinis GDL (f) (RRB)	26	+	+	+	+	+	+	+	+	−	+	−	−
Murine													
M. neurolyticum type A (f) (RRB)	102	+	+	+	+	+	ND	ND	+	−	+	ND	ND
M. arthritidis PG6 (n) (RRB)	644	−	−	−	+	+	+	+	+	−	+	−	−
Feline													
M. gatae KDC (n) (BB)	28	ND	−	−	−	+	+	+	+	−	−	ND	ND
Avian													
M. gallisepticum PG31 (f) (RRB)	21	+	+	+	+	+	ND	+	+	−	ND		
M. anatis 1340 (f) (BB)	102	+	+	+	+	+	+	+	+	−	−	ND	ND
M. meleagridis 17529 (n) (RRB)	85	+[h]	−	−	+	+	+	+	+	−	+	−	−
Acholeplasma spp.													
A. laidlawii PG8 (f) (RRB) PG9 (f) (RRB)	ND	+	+	+	+	−	+	+	+	+	+	+	+
A. granularum BTS39 (f) (RRB)	98	+	+	+	+	+	+	+	+	+	+	+	+
A. axanthum S743 (f) (JT)	16	+	+	+	+	−	+	+	+	+	+	+	+
A. oculi 19L (f) (JT)	69	+	+	+	+	+	+	+	+	+	+	+	+
A. modicum PG49 (f) (JT)	80	+	+	+	+	+	+	+	+	+	+	+	+

[a] Protein concentration of crude extract added to gel in 35-μl aliquots. Histochemical stain was applied after electrophoresis, and the presence of visible stain deposition in a gel lane was scored as +. Although the technique is not strictly quantitative, a negative test indicates an approximate level of less than 1% of the activity measurable in + extracts as determined by serial dilution of + extracts (data not shown) (25). Relative mobilities of isozymes are illustrated in Fig. 2.

[b] TPI, Triose phosphate isomerase; G3PD, glyceraldehyde-3-phosphate dehydrogenase; GPI, glucose phosphate isomerase; NP, purine nucleoside phosphorylase; PP, inorganic pyrophosphatase; AK, adenylate kinase; DIP, dipeptidase; ESA, esterase; SOD, superoxide dismutase; ACP, acid phosphatase; G6PD, glucose-6-phosphate dehydrogenase; PGD, phosphogluconate dehydrogenase.

[c] (f) Glucose fermenter; (n) glucose nonfermenter, arginine utilizer.

[d] (RRB) Obtained from the Reference Reagents Branch, National Institute of Allergy and Infectious Diseases; (BB) triple-filtered clone purified at the Bureau of Biologics, National Institutes of Health; (JT) obtained from J. G. Tully, Bethesda, Md.

[e] ND, Not done.

[f] Trace activity of glucose phosphate isomerase was observed in one strain of *M. hominis* (1105). Four other strains were negative (PG21, H34, 1257, and 760).

[g] Trace activity of superoxide dismutase was seen in *M. faucium*. The activity in these extracts was 10- to 100-fold less than activity observed in the *Acholeplasma* species.

[h] Trace activity of triose phosphate isomerase was seen in *M. meleagridis*.

TABLE 3. *Mycoplasma isozyme expression in infected-cell lysates and in particulate cell exudates*

Cell line	Strain	Titer ($\times 10^{-7}$)[a]	Enzyme expression[b]						
			TPI	GPI	NP-AP	DIP	AK	ACP	G3PD
HEp-2	*A. laidlawii* PG8	15	C,F	C,F	C	–	–	–	–
	M. arginini G230	12	–	–	–	F	–	–	–
	M. arthritidis PG27	45	–	–	–	–	–	–	–
	M. faucium DC333	ND	–	–	–	–	–	–	–
	M. fermentans PG18	45	–	–	–	–	–	–	–
	M. gallisepticum PG31	15	–	–	–	–	–	–	–
	M. hominis 1185	600	C	C	C,F	–	–	F	–
	M. hyorhinis DBS1050	1,000	C,F	C	F	–	–	–	F
	M. hyorhinis PG29	600	C,E	C,E	–	–	–	–	–
	M. neurolyticum type A	12	–	–	–	–	–	–	–
	M. pulmonis Ash	600	C	C	–	–	–	–	–
	M. salivarium PG20	45	–	–	–	–	–	–	–
	M. orale 1550	600	–	C	–	–	–	–	–
L cell	*A. laidlawii* PG8	1,000	C,F	C,F	C	F	F	C	–
	M. arginini G230	100	–	–	C	–	F	–	–
	M. arthritidis PG27	6	–	–	–	C	–	C	–
	M. capricolum 14	150	–	–	–	–	–	–	–
	M. gallisepticum PG31	6	–	–	–	–	–	–	–
	M. hominis 1185	600	–	–	–	–	–	–	–
	M. hyorhinis DBS1050	1,000	C,F	C,F	–	–	–	–	C,F
	M. hyorhinis PG29	1,000	F	C,F	–	–	–	–	–
	M. neurolyticum type A	12	C	–	–	–	–	–	–
	M. pulmonis Ash	600	C,F	C	F	–	–	–	–
	M. salivarium PG20	6	–	–	–	–	–	–	–
	M. orale 1550	600	–	–	–	–	–	–	–
Vero	*M. hyorhinis* DBS1050	1,000	C,F	C,F	C	C,F	C	C	C,F

[a] Titer of mycoplasma per infected-cell culture was determined by CFU and CCU (see the text).

[b] TPI, Triose phosphate isomerase; GPI, glucose phosphate isomerase; NP-AP, Purine nucleoside phosphorylase-adenosine phosphorylase; DIP, dipeptidase; AK, adenylate kinase; ACP, acid phosphatase; G3PD, glyceraldehyde-3-phosphate dehydrogenase; C, Enzyme observed in detergent-treated cell lysates; F, enzyme observed in concentrated particulate cell fluids; –, enzyme not detected (see the text for extraction procedures). No activity specific for mycoplasmas was seen in infected-cell preparations with the enzymes esterase, inorganic pyrophosphatase, or superoxide dismutase. Cell extracts and fluids were not tested for glucose-6-phosphate and 6-phosphogluconate dehydrogenases.

tity of the infecting mycoplasma as well as the particular mammalian cell under investigation.

Five enzyme systems were observed in both cell lysates and in cell fluid concentrates. The mycoplasma enzymes which showed the highest activity in infected cultured cells determined by limiting dilution (25) were triose phosphate isomerase, glucose phosphate isomerase, and nucleoside phosphorylase. Consequently, these three enzymes represent the best candidates for detecting mycoplasmas in infected cells. However, the usefulness of these enzymes is limited somewhat by their distribution in various mycoplasmas (Table 2) as well as by coincidental mobilities of homologous enzymes produced by the host cells and the infecting mycoplasma.

DISCUSSION

More than 100 gene-enzyme systems have been described in biochemical and genetic studies of mammalian cells (14). We have chosen to examine 22 mycoplasmas, including the most common contaminants of cell cultures (5), for the presence of 21 enzymes homologous to mammalian isozymes. Broth-grown mycoplasmas and mycoplasma-infected mammalian cell cultures were examined. Twelve isozyme systems were easily detected in some or all mycoplasmas, whereas nine enzymes were not detected in extracts of 20 mycoplasmas tested (Table 1). Of the 12 enzymes found in mycoplasmas, 9 (purine nucleoside phosphorylase, adenylate kinase, dipeptidase, esterase, glyceraldehyde-3-phosphate dehydrogenase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and superoxide dismutase) have been reported previously for selected species (16, 22, 32, 34, 43; M. Gerwitz and P. J. Van DeMark, Bacteriol. Proc. **45**:77, 1966; G. Y. Lee and G. E. Kenny, Program Abstr. Int. Org. Mycoplasmol. 3rd., Custer, S.D., p. 39, 1980; R. M. Lemcke, S. M. Lanham, and C. M. Scott, Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **241**:10, 1978). To our knowledge, the remaining three enzymes (triose phosphate isomerase, inorganic pyrophosphatase, and acid phosphatase) have not been re-

ported in mycoplasmas. The relative electrophoretic mobilities of the 12 enzymes were characteristic for each mycoplasma (Fig. 2) and could serve as a useful biochemical parameter for identification and classification of genetically divergent species. Our results augment the findings of Lanham et al. (16) who examined multiple strains of two *Acholeplasma* species for 15 isozyme systems. The sum of our results, those of Lanham et al. (16), and others (1, 33, 34; Lee and Kenny, Program Abstr. Int. Org. Mycoplasmol. 3rd, Custer, S.D., p. 39, 1980) extend the number of described isozymes in mycoplasmas to over 25 systems. Similar technology could readily be applied to numerous other soluble protein and enzyme systems described in mycoplasmas (9, 19, 22, 31, 32, 36, 37, 40–43).

An interesting aspect of these data is the exclusive appearance of three isozyme systems (superoxide dismutase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase) in *Acholeplasma* species. The single exceptional *Mycoplasma* species (*M. faucium*) expresses only trace amounts of superoxide dismutase (less than 1% of activity seen in *Acholeplasma* species). The restriction of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase to *Acholeplasma* species suggests that the pentose shunt is operative in this genus, but not in *Mycoplasma*. The strict concordance of detection of these three enzymes presents itself as a useful and rapid diagnostic characteristic of the *Acholeplasma* genus.

The detection of substantial levels of three glycolytic enzymes, glucose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and triose phosphate isomerase, in each of the fermenting mycoplasmas and their absence in nonfermenting species supports the classification scheme of fermenting versus nonfermenting mycoplasmas (38). The detection of trace levels of glycolytic enzymes in *M. meleagridis* and *M. hominis* was an unexpected observation which cannot be understood with the available data.

The reactive superoxide radical O_2^- is a minor, but not trival, product of biological oxidation of molecular oxygen (13). Fluxes of O_2^- have been shown to be cytotoxic and may participate in mycoplasma cytopathic effects. Superoxide dismutase catalyzes the dismutation of O_2^- to form hydrogen peroxide, a biological product of a number of species, including *M. pneumoniae*, *M. hyorhinis*, *M. neurolyticum*, *M. arthritidis*, *M. gallisepticum*, *A. laidlawii*, *A. granularum*, and *A. oculi* (8, 10, 11). Several other species (*M. hominis*, *M. fermentans*, and *M. salivarium*) failed to produce peroxide in parallel assays (11). Our results do not show a correlation between peroxide production and superoxide dismutase activity. Apparently, those strains which produce peroxide but lack superoxide dismutase activity utilize a different mechanism for generation of excreted peroxide.

Two enzymes involved in purine nucleoside metabolism, nucleoside (inosine) phosphorylase and adenylate kinase, were also detected. We have reported elsewhere (manuscript in preparation) that nucleoside phosphorylase activity is carried by the same protein that catalyzes the adenosine phosphorylase activity which was reported by Hatanaka and co-workers (15, 37, 40) and found to be characteristic of and restricted to mycoplasmas (15, 37, 40). The remaining four enzymes (inorganic pyrophosphatase, dipeptidase, esterase, and acid phosphatase) were detected with nonphysiological substrates, which makes identification of their metabolic role difficult.

A number of enzymes were present in multiple forms or isozymes in certain species (Fig. 2). Since the extracts were prepared from clone-purified cultures, genetic polymorphism is an unlikely explanation. A number of possible biochemical mechanisms for isozyme formation have been discussed in detail elsewhere (21, 29) and include posttranslational enzyme modification (e.g., phosphorylation and sialic acid addition), different interaction with cofactors, heteromultimeric enzymes, multiple structural genes for enzymes with the same activities, and conformational isozymes. The biochemical basis of isozyme formation seen in mycoplasmal enzymes is unclear at present and must await biochemical or genetic analysis or both.

Among the enzymes we failed to detect were malate dehydrogenase, isocitrate dehydrogenase, and malic enzyme. The first two enzymes participate in the tricarboxylic acid (Krebs) cycle, and the third enzyme is indirectly involved in malate regulation. The absence of the three enzymes has been reported in *Acholeplasma* species (31) and is consistent with a lack of a tricarboxylic acid cycle in mycoplasmas which are thought to be deficient in the apparatus of oxidative phosphorylation (31). Hexosaminidase and glyoxylase, not generally present in procaryotes, were also absent in our study. Although it is tempting to conclude that a negative result indicates absence of the enzyme, we cannot exclude the interpretation that the enzyme is present in the mycoplasma but not resolved under the conditions used which were optimal for the mammalian enzymes. No attempt was made to adjust reaction conditions to those of previously reported biochemical characteristics of mycoplasmas and bacterial enzymes.

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