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On the Distribution and Characteristics of Isozyme Expression in *Mycoplasma, Acholeplasma,* and *Ureaplasma* Species

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A summary of a survey of three genera of mycoplasmatales (*Mycoplasma, Acholeplasma,* and *Ureaplasma*) for isozyme expression is presented. Isozyme analysis of mycoplasmas has been employed in at least three distinct areas: (*1*) as genetic markers for identification, individualization, and taxonomic classification; (*2*) as markers for cell culture contamination; and (*3*) as a qualitative measure of the operative metabolic pathways in the diverse species. We have found five ubiquitous enzymes: purine nucleoside phosphorylase, adenylate kinase, inorganic pyrophosphatase, dipeptidase, and esterase. Three enzymes, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and superoxide dismutase, were restricted to *Acholeplasma* species and were not detected in *Mycoplasma* or *Ureaplasma.* Four glycolytic enzymes, glucose phosphate isomerase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase, were restricted to those species of *Mycoplasma* and *Acholeplasma* capable of glucose fermentation. Two of these glycolytic enzymes, glucose phosphate isomerase and lactate dehydrogenase, were detected in serovars I and II of *U. urealyticum,* which is inconsistent with the non-glycolytic activity in this genus.

A few years ago, we initiated a collaborative effort to study the occurrence and characteristics of isoenzyme expression in a number of mycoplasma species [1]. Over 100 histochemical enzyme stains had been developed for studies in mamalian, insect, prokaryote, and lower eukaryotic biological species. Studies of these isozyme systems have resulted in a variety of advances in biochemistry, development, genetics, and evolution [2-4]. The analysis of mycoplasma species using these same technologies held some promise from at least three biological perspectives. Our potential goals were: first, to develop biochemical genetic characteristics for use in identification and classification of established mycoplasma species as well as of newer isolates; second, to evaluate the use of mycoplasma isozymes as specific markers for detection of cell culture contamination: and third, to contribute, when possible, to the understanding of cellular metabolism of these fascinating organisms by the detection of specific enzymes which are included in well-defined metabolic pathways of importance in other biological systems.

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In this report, we will summarize our findings in surveys of three mycoplasmatales genera (*Mycoplasma, Acholeplasma,* and *Ureaplasma*) from each of these perspectives. In general, the isozyme techniques have been used for the study of each genus and, to date, over 30 different enzymes have been detected in the various species examined. The pattern of expression (whether a species or strain possesses an activity or not) do appear to differ between genera (e.g., *Acholeplasma* vs. *Mycoplasma*) and the mobilities of several isozymes are sufficiently unique to constitute a genetic "signature" for different species [5]. Infected mammalian cells often express mycoplasma isozymes; however, the use of isozyme analysis as a diagnostic tool has certain limitations [1]. We have examined two serotypes of *Ureaplasma urealyticum* for the presence of a battery of enzymes previously observed in *Acholeplasma* and/or *Mycoplasma* species. The enzymes studied are members of specific metabolic pathways suspected of contributing to energy-yielding production in the species. The results of the ureaplasma study excluded the presence of certain pathways (such as arginine hydrolysis, the purine salvage pathway, and the pentose shunt) while certain glycolytic enzymes were detected. The presence of glycolytic enzymes in ureaplasmas was unexpected and may provide an important clue to alternate mechanisms for deriving energy.

MATERIALS AND METHODS

Growth Conditions

The strains and culture conditions of broth-grown *Mycoplasma* and *Acholeplasma* species have been described [1]. *Ureaplasma urealyticum* serovars I and II (obtained from M.C. Shepard, Camp LeJeune, NC) were grown in five-liter batches of PPLO broth (Difco) supplemented with 10 percent unheated horse serum, 5 percent fresh yeast extract (Flow Laboratories, McLean, VA) 7 mM urea, and 1,000 units/ml of penicillin G. To reduce the amount of precipitable material in the culture medium, the serum and yeast extract components were centrifuged at 12,000 *g* for 30 minutes and the resulting supernatant fluids were filtered through a 450 nm pore-diameter membrane filter before being added to the basal medium. The cultures were harvested after 16–20 hours of incubation at 37°C, when the pH of the medium reached a value of about 7.4 (initial pH was about 6.5). The organisms were harvested by centrifugation at 12,000 *g* for 30 minutes in the cold, and the pellet was washed once in 0.25 M NaCl. *Mycoplasma* and *Acholoplasma* extracts for isozyme analysis were prepared as previously described [1].

Isoenzyme Electrophoresis and Stain Development

The mycoplasma extracts were subjected to starch gel electrophoresis, as described previously [1,6]. After electrophoresis, the gels were sliced horizontally and stained histochemically for a number of enzymes previously detected in certain strains and species of *Mycoplasma* and *Acholeplasma* [1]. In addition, four other isozyme systems were examined: arginine deiminase (ADI), carbamoyl phosphokinase (CPK), lactate dehydrogenase (LDH), and mannose phosphate isomerase [2,7]. Urease activity was measured by the disappearance of radioactive urea added to the cell extract [8]. [^{14}C] urea with a specific activity of 56.0 mCi/m mole was purchased from New England Nuclear, Boston, MA. The labeled urea was added to a stock solution of 1 M urea in water, to give a final concentration of 1 μCi/ml of [^{14}C] urea. The tested cell extract (50μl) was diluted in 2 ml of phosphate-buffered saline, pH 5.9 and 50 μl of the labeled urea solution were added. The reac-

tion mixture was incubated at 37°C and duplicate 0.25 ml samples were withdrawn at 0, 15, 30, and 90 minutes, and transferred to scintillation vials containing 0.5 ml of 3.6N H_2SO_4. The vials were kept at room temperature for at least 60 minutes to stop enzymatic activity and to facilitate the removal of solubilized radioactive CO_2. Ten ml of Aquasol scintillation cocktail (New England Nuclear, Boston, MA) were added to each vial and radioactivity was determined in a liquid scintillation spectrometer.

RESULTS

In a survey of 22 species of *Acholeplasma* and *Mycoplasma,* twelve isozyme systems were detected in some or all of the species examined [1]. One of the enzymes, glyceraldehyde-3-phosphate dehydrogenase (G3PD), is illustrated in Fig 1. This enzyme is a member of the glycolytic pathway and, as might be expected, was found only in those species of mycoplasma which were capable of glucose fermentation. Two other glycolytic enzymes, triose phosphate isomerase and glucose phosphate isomerase, were likewise concordant in their expression with G3PD and glucose fermentation. Conversely, these three enzyme activities were not found in species which were nonfermenters and which generate energy by arginine hydrolysis [9].

Five enzymes [purine nucleoside phosphorylase (NP), adenylate kinase (AK), inorganic pyrophosphatase (PP), dipeptidase (DIP), and esterase (EST)] were ubiquitous within our sampling of *Mycoplasma* and *Acholeplasma* species. The mobility of purine nucleoside phosphorylase (NP) is presented in Fig. 2A. Nucleoside phosphorylase activity in certain species is of particular importance in mycoplasma-infected cell cultures. Because the enzyme is very active and widely distributed in mycoplasma species, NP is readily detected in infected mammalian cell cultures (Fig. 2B). Furthermore, we have shown that mycoplasma NP has two catalytic activities, inosine phosphorylase *and* adenosine phosphorylase [10]. The dual activity has not been seen in the mammalian NP, which is not reactive to exogenous adenosine. Adenosine phosphorylase activity has been shown by Hatanaka and co-workers [11,12] to be diagnostic for mycoplasma contamination of cell cultures. More recently, McGarrity and Carson [13] have described a method for detecting mycoplasma infection of cell cultures by exposing cultured cells to a purine analogue

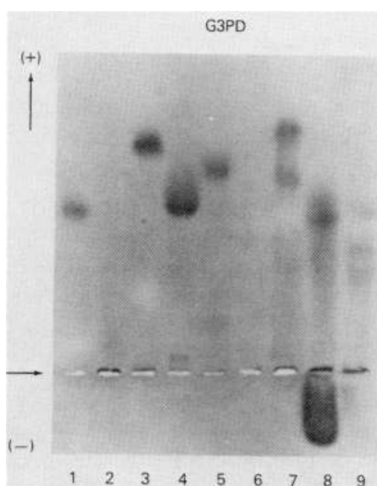

FIG. 1. Electropherogram of glyceraldehyde-3-phosphate dehydrogenase (G3PD) in broth-grown mycoplasmas. (1) *A. modicum;* (2) *M. salivarium;* (3) *A. laidlawii;* (4) *M. anatis;* (5) *M. gallisepticum;* (6) *M. arginini;* (7) *A. granularum;* (8) *M. neurolyticum;* (9) *M. conjunctivae.*

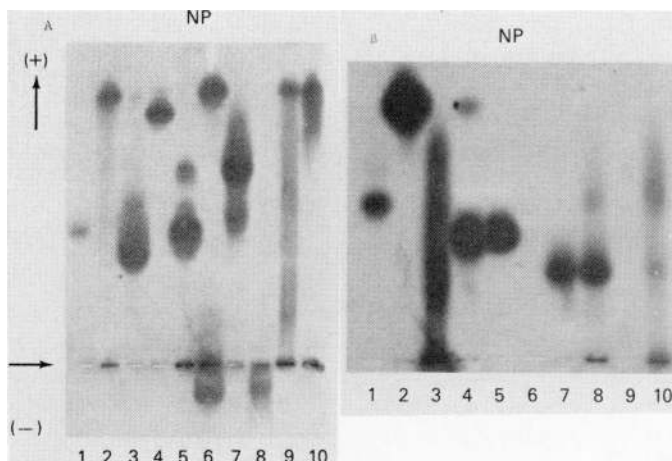

FIG. 2. Electropherogram of purine nucleoside phosphorylase (NP) in broth-grown mycoplasmas (A). **(1)** HeLa; **(2)** *A. modicum;* **(3)** *M. anatis;* **(4)** *M. hominis;* **(5)** *M. arthritidis;* **(6)** *M. gallisepticum;* **(7)** *M. meleagridis;* **(8)** *M. orale;* **(9)** *A. oculi;* **(10)** *M. arginini.* B. nucleoside phosphorylase in mycoplasmas and in infected tissue culture cells. **(1)** *M. salivarium;* **(2)** *M. hominis;* **(3)** *M. hyorhinis;* **(4)** HeLa cell infected with *M. hominis;* **(5)** uninfected HeLa cell; **(6)** concentrated (by centrifugation) cell fluid of HeLa infected with *M. hominis;* **(7)** uninfected Vero (African green monkey) cells; **(8)** Vero cell infected with *M. hyorhinis;* **(9)** concentrated cell fluid from uninfected Vero cells; **(10)** concentrated cell fluid from *M. hyorhinis*-infected Vero. Note that the smear in lane 3 is an artefact due to overloading. The mobility of this enzyme is identical to that of *M. salivarium* (lane 1, [1]).

6-methyl purine deoxyriboside. Infected cells convert this to the cytotoxic antimetabolites 6-methyl purine and 6-methyl purine riboside by catalysis of the mycoplasma adenosine phosphorylase (which is actually nucleoside phosphorylase). Thus, a rapid growth assay for mycoplasma-infected cells is indicated.

Three enzymes (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and superoxide dismutase) were found in abundant activity in each of five *Acholeplasma* species (*A. laidlawii, A. granularum, A. axanthum, A. oculi,* and *A. modicum*) but were not found in any of the 15 *Mycoplasma* species examined, which prompted us to suggest that these enzymes might be specific and characteristic of *Acholeplasma* species. In recent analyses (Table 1) of some newer unclassified plant *Acholeplasma* isolates, four strains (PP1, GF1, W20, and W24) were found to lack all three enzyme activities while the same extracts were highly active in more ubiquitous enzyme systems such as nucleoside phosphorylase, dipeptidase, and glucose phosphate isomerase. However, one of the new plant isolates (*Achleplasma florum* strain L1) was positive for the three *Acholeplasma*-specific enzymes. These results may be important in the classification and differentiation of *Acholeplasma* species derived from plant tissues since they reflect in a modest way the physiological similarity (or dissimilarity) among these strains.

Ureaplasma urealyticum has been implicated as the etiologic agent in recurrent nongonococcal urethritis and, as such, is an important potential human pathogen

[14]. The genus has at least nine serologically defined groups, which can be divided into two clusters based upon restriction enzyme patterns of digested genomic DNA [15]. The *Ureaplasma* genus is characterized by its ability to hydrolyze urea due to a large amount of urease enzyme activity [16]. Yet, very little is known about the pathogen's energy metabolism. We examined extracts of two serovars (I and II), one belonging to each cluster, for expression of a number of isozymes previously seen in the *Mycoplasma* and *Acholeplasma* species. The analysis was appreciably complicated by the fastidious growth characteristics of *U. urealyticum.* These organisms grow to very low titers, are sensitive to alkaline pH, and tend to nonspecifically bind horse serum proteins present in the growth media. Thus, large pellets of *U. urealyticum* were often grossly contaminated with serum protein. For these reasons, positive enzyme activities were considered valid only when they (*1*) had a different electrophoretic mobility from the homologous enzyme in horse serum, and (*2*) showed different mobilities between serovars. Negative enzyme activity was considered valid when the same extracts showed appreciable activities of the ureaplasma-positive enzymes. Nonetheless, negative enzyme scores were always subject to some degree of uncertainty for reasons discussed previously [1].

The results of our survey of *U. urealyticum* serovars I and II are presented in Table 2 and illustrated in Fig. 3. In summary, four enzymes, glucose phosphate isomerase, lactate dehydrogenase, nucleoside phosphorylase, and dipeptidase were detected in these agents. All of the other enzymes (Table 1), commonly seen in abundant quantities in other mycoplasmas, were not detected in the ureaplasma serovars. Among the negatives were enzymes of the pentose shunt (G6PD and PGD), enzymes in the arginine hydrolysis pathway (carbamoyl phosphokinase and arginine

TABLE 1
Isozyme Expression in *Acholeplasma* Species

Acholeplasma species, strain	Presence (+) or absence (−) of isozyme system (*)					
	DIP	NP	GPI	G6PD	PGD	SOD
A. axanthum, S743	+	+	+	+	+	+
A. equifetale, C112	+	+	+	+	N.D.	+
A. granularum, BTS39	+	+	+	+	+	+
A. hippikon, C1	+	+	+	+	N.D.	+
A. laidlawii, PG8	+	+	+	+	+	+
A. morum, S2	+	+	+	+	+	+
A. modicum, PG-49	+	+	+	+	+	+
A. oculi, 19L	+	+	+	+	+	+
A. fluoridum, L1	−	+	+	+	+	+
PP1	+	+	+	−	−	−
GF1	+	+	+	−	−	−
W20	+	+	+	−	−	−
W24	+	+	+	−	−	−
HeLa cell culture	+	+	+	+	+	+

*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 percent of the activity measurable in + extracts as determined by serial dilution of + extracts (data not shown). DIP, dipeptidase; NP, purine nucleoside phosphorylase, GPI, glucose phosphate isomerase; G6PD, glucose-6-phosphate dehydrogenase; PGD, phosphogluconate dehydrogenase; SOD, superoxide dismutase.

N.D., not done.

TABLE 2
Survey of *U. urealyticum* for Isozymes of Potentially Important Metabolic Pathways

Metabolic Pathway	Diagnostic Enzyme	Status in *U. urealyticum**
I Glycolysis	Glucose phosphate isomerase	+
	Triose phosphate isomerase	–
	Glyceraldehyde-3-phosphate dehydrogenase	–
	Lactate dehydrogenase	+
II Arginine hydrolysis	Arginine deiminase	–
	Carbamoyl phosphokinase	–
III Acetate kinase	Acetate kinase	N.D.
IV Purine salvage pathways	Adenine phosphoribosyl transferase	–
	Hypoxanthine phosphoribosyl transferase	–
	Purine nucleoside phosphorylase	+
V Pentose shunt	Glucose-6-phosphate dehydrogenase	–
	6-Phosphogluconate dehydrogenase	–
VI Phosphatases	Inorganic pyrophosphatase	–
	Acid phosphatase	–
VII Other	Nonspecific esterase	–
	Dipeptidase	+
	Superoxide dismutase	–

N.D., Not determined

*Ureaplasma extracts were prepared by sonication in 0.05 M Tris HCl, pH 7.1, 1 mM EDTA, 1 percent Triton X-100 [1]. All extracts hydrolyzed 50 percent of 1 M urea in ≤ 15 minutes as described in Materials and Methods.

deiminase), superoxide dismutase, esterase, and two phosphatases. Two of the enzymes (GPI and LDH) present in ureaplasmas are active at the beginning and end of glycolysis, respectively. However, it was perplexing to find that two other glycolytic enzymes [triose phosphate isomerase (TPI) and glyceraldehyde-3-phosphate dehydrogenase (G3PD)] were not detected in these serovars. Thus, it is not clear whether glycolysis is functional (with possibly technical difficulties, precluding our detecting a functional G3PD and TPI) or whether the pathway is only partially intact or even vestigial. The resolution of this dilemma is under investigation.

DISCUSSION

The use of isozyme technologies to investigate the mycoplasma species offers a useful approach to a number of basic questions in the field. Abundant isozymes (such as nucleoside phosphorylase, glucose phosphate isomerase, and triose phosphate isomerase) are easily detected in infected cell cultures. However, the usefulness of these enzymes is limited somewhat by their distribution in various mycoplasmas as well as by coincidental mobilities of homologous enzymes produced by the host cells and the infecting mycoplasma [1].

The failure to detect certain enzymes in otherwise active extracts of *Ureaplasma urealyticum* argues against the existence of certain enzymatic pathways involved in energy production (arginine hydrolysis, the pentose shunt, and purine salvage pathways). The interpretation of the role of glycolysis is not immediately apparent,

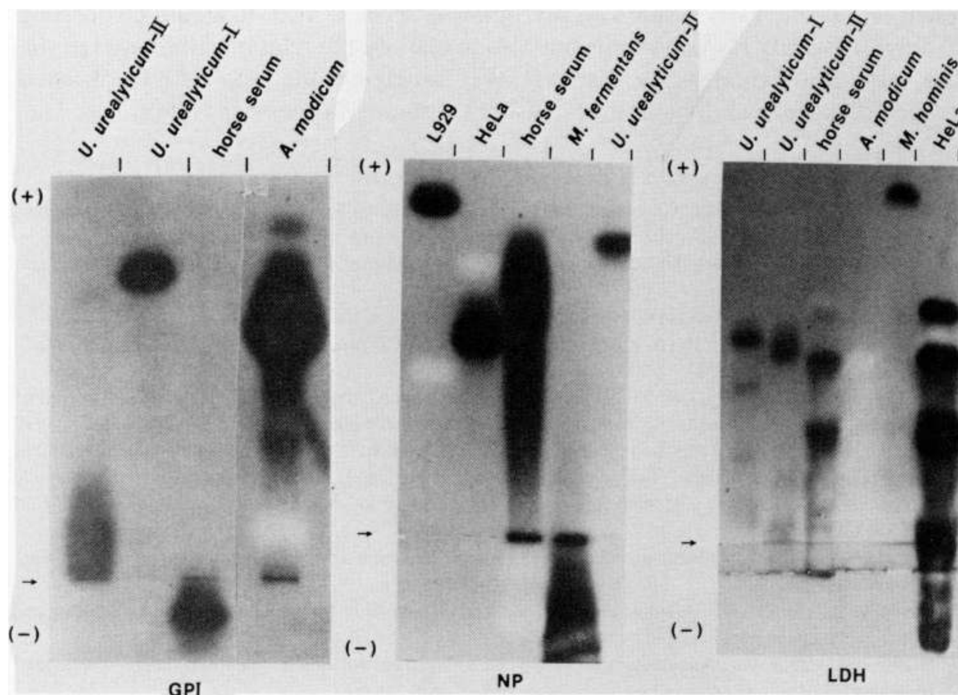

FIG. 3. Electropherograms of three enzyme stains with extracts of *Ureaplasma urealyticum* serovar I and II, cultured mammalian cells, human HeLa and mouse L929, horse serum, and certain *Mycoplasma* and *Acholeplasma* species. The enzymes were glucose phosphate isomerase (**GPI**), purine nucleoside phosphorylase (**NP**), and lactate dehydrogenase (**LDH**).

since two glycolytic enzymes (GPI and LDH) were detected and two (TPI and G3PD) were not. It may be important to note that two enzymes which were detected are among the strongest of mycoplasma enzymes in our battery, while the two we failed to detect are among the weakest. In light of the fastidious growth characteristics of ureaplasmas and their tendency to bind serum proteins, it is tempting to suspect that the two missing activities were due to technical problems and that glycolysis may be intact in this genus. Confirmation of this hypothesis must await further metabolic analyses.

A major advantage of isozyme technologies in mammalian systems stems from the accumulation of amino acid substitutions in homologous proteins during evolutionary divergence. Physiologically trivial amino acid substitutions which alter the net charge of the protein (but not the catalytic function) have the result of altering the electrophoretic mobility of these proteins. An important aspect of this process is the tendency of these substitutions to occur at a constant rate during evolution. Amino acid substitutions can be used as an evolutionary "clock" which "ticks" away through evolutionary time [17]. Because of the relative constancy of the clock, the cumulative substitutions which occur at a large number of homologous loci can be used as a metric of "genetic distance" [18,19]. Thus, the comparative analysis of a group of 20 or more homologous isozyme systems has been used to assess the time which has elapsed since two extant species became reproductively isolated. The genetic distance statistic is very useful for generating phylogenetic topologies which can represent a variety of genera and species within a biological family. With the

present and future isozyme data on mycoplasma species, then, it should be possible not only to identify a species unit, but also to classify the relationships between the units from an evolutionary context. We anticipate the application of such methodologies to questions in mycoplasma taxonomy in the near future.

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