Localization of Enzymes in *Mycoplasma*¹

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ABSTRACT

POLLACK, J. D. (University of Connecticut, Storrs), SHMUEL RAZIN, AND ROBERT C. CLEVERDON. Localization of enzymes in *Mycoplasma*. J. Bacteriol. 90:617-622. 1965.—Cells of eight parasitic and two saprophytic *Mycoplasma* strains were lysed by use of osmotic shock, and the membranes were separated from the soluble fraction by use of differential centrifugation. Cell fractions were tested for reduced nicotinamide adenine dinucleotide (NADH₂) oxidase, reduced nicotinamide adenine dinucleotide phosphate (NADPH₂) oxidase, glucose-6-phosphate dehydrogenase, adenosine triphosphatase, ribonuclease, and deoxyribonuclease activities. Adenosine triphosphatase was confined to the membrane fraction of all *Mycoplasma* strains. The NADH₂ oxidase activity was associated with the membranes of the saprophytic *M. laidlawii* and with the soluble fraction of the parasitic *Mycoplasma* strains. NADPH₂ oxidase activity was detected only in the soluble fraction of the parasitic strains. Glucose-6-phosphate dehydrogenase was demonstrated only in the soluble fraction of *M. laidlawii*. Ribonuclease activity was found usually in both membrane and soluble fractions, but was generally higher in the membrane fraction. In the human and bovine *Mycoplasma* strains, deoxyribonuclease activity could not be demonstrated in the soluble fraction; in the remaining strains, activity was highest in the soluble fraction. Dissolution of *M. laidlawii* strain B membranes by sodium deoxycholate significantly increased membrane-NADH₂ oxidase and adenosine triphosphatase activities.

The internal organization and the location of enzymes in *Mycoplasma* cells is of special interest, because *Mycoplasma* species are the smallest organisms capable of autonomous growth. *Mycoplasma*, being devoid of cell walls and sensitive to osmotic lysis without any prior treatment (Razin, 1963, 1964), is especially suited for enzyme localization studies.

A preliminary study (Pollack et al., 1965) was carried out with two *Mycoplasma* strains, the saprophyte *M. laidlawii* B and the caprine *Mycoplasma* 14. Adenosine triphosphatase activity was confined to the membrane in both strains, whereas reduced nicotinamide adenine dinucleotide (NADH₂) oxidase activity was confined to the membrane of *M. laidlawii* and to the soluble fraction of *Mycoplasma* 14.

The results of the present investigation show the distribution between membrane and soluble fractions of NADH₂ and reduced nicotinamide adenine dinucleotide phosphate (NADPH₂)

oxidases, adenosine triphosphatase, glucose-6-phosphate dehydrogenase, ribonuclease, and deoxyribonuclease activities in 10 representative *Mycoplasma* strains. The results obtained provide data useful for the comparison of the organization of *Mycoplasma* cells with that of other microorganisms, and for the classification of strains within the *Mycoplasma* group.

MATERIALS AND METHODS

Cultures. The two saprophytic strains, *M. laidlawii* A (PG8) and *M. laidlawii* B (PG9), as well as the parasitic *M. neurolyticum* (PG28, murine) and *M. bovigentalium* (PG11, bovine), were obtained from D. G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent, England). *M. gallisepticum* S6 (avian) and *Mycoplasma* sp. 14 (caprine) were obtained from H. E. Adler (School of Veterinary Medicine, University of California, Davis). *M. hominis* ATCC 14152 (human) and *M. arthritidis* ATCC 14124 (human, rat) were kindly provided by R. G. Wittler (Walter Reed Army Institute of Research, Washington, D.C.). *M. gallisepticum* A5969 (avian) was obtained from M. E. Tourtellotte (Department of Animal Diseases, University of Connecticut, Storrs). *Mycoplasma* sp. H39 (human) was obtained from P. F. Smith (Department of Microbiology, School of Medicine, University of South Dakota, Vermillion).

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Micrococcus lysodeikticus was obtained from the culture collection of the Department of Bacteriology, University of Connecticut.

Media and growth conditions. *Mycoplasma* strains were grown in a modified liquid Edward medium (Razin, 1963). Swine serum (Flow Laboratories, Baltimore, Md.) replaced PPLO Serum Fraction (Difco) for the growth of *M. gallisepticum* A5969. For some experiments, *Mycoplasma* strains were grown in Tryptose broth consisting of (per liter): Tryptose (Difco), 20 g; NaCl, 5 g; tris-(hydroxymethyl)aminomethane (Tris), 5 g; glucose, 10 g; PPLO Serum Fraction, 10 ml. The pH of this medium was 8.2 to 8.4 without adjustment.

Broth cultures were grown statically for 24 hr at 37 C in Corning low-form flasks containing 1.5 liters of medium which had been inoculated with 100 ml of a 24-hr culture.

Micrococcus lysodeikticus was grown on Brain Heart Infusion Agar (Difco) for 24 hr at 37 C.

Preparation of cellular fractions. *Mycoplasma* cells were harvested by centrifugation at $9,000 \times g$ for 30 min and washed twice by 250-fold dilution in "B2" buffer containing the following: 0.156 M NaCl, 0.050 M Tris, 0.010 M 2-mercaptoethanol in deionized water, adjusted to pH 7.4 with HCl. The cells were examined for osmotic fragility by use of the criteria described by Razin (1963, 1964). The washed sedimented cells (about 0.5 to 1.5 g, wet weight) were suspended in 30 to 50 ml of "B2" buffer diluted 1:20 in deionized water, and were incubated at 37 C for 30 min. The treated suspensions were centrifuged for 5 to 8 min at $8,000 \times g$ to remove clumps and unbroken cells. The supernatant fraction (whole lysate) was carefully decanted and centrifuged for 40 min at $37,000 \times g$ to collect membranes. The membranes were washed twice by use of 500-fold dilution in 1:20 "B2" buffer. The supernatant fluid from the membrane collection was centrifuged for 2 hr in a Spinco 40 head at 40,000 rev/min to sediment the ribosomes and other particulate matter. The supernatant fluid from the ribosome collection was designated as the soluble fraction. All centrifugations were conducted at 5 C.

Protoplasts of *M. lysodeikticus* were prepared by use of lysozyme treatment according to Gilby and Few (1960). The protoplasts were osmotically lysed in 1:20 "B2" buffer containing 0.1 $\mu\text{g}/\text{ml}$ of crystalline deoxyribonuclease. The membrane and soluble fractions of the protoplast lysate were separated as described for *Mycoplasma*.

Analytical methods. NADH₂ and NADPH₂ oxidase activities were measured spectrophotometrically by following the decrease in absorbance at 340 m μ upon addition of NADH₂ and NADPH₂ at 34 C. The reaction mixture (3.0 ml) contained 0.005 to 1.63 mg of cell protein (dictated by activity), and the following (in micromoles): NaCl, 23.4; Tris-HCl (pH 7.4), 7.5; 2-mercaptoethanol, 1.5. The reaction was started with 3.6 μmoles of NADH₂ or NADPH₂. Data were expressed as the decrease in absorbance at 340 m μ per minute per milligram of protein.

Adenosine triphosphatase activity was measured by the release of inorganic phosphorus in reaction mixtures incubated at 37 C. The reaction mixture (1.0 ml) contained 0.069 to 1.45 mg of cell protein (dictated by activity), and the following (in micromoles): MgCl₂, 1.25; NaCl, 1.1; Tris-HCl (pH 8.0), 50.0; 2-mercaptoethanol, 0.6. The reaction was started with 1.25 μmoles of adenosine triphosphate (ATP). Controls lacked either cell protein or ATP. At four to eight intervals over 30 min, 1.0 ml of cold 10% trichloroacetic acid was added to stop the reaction. Inorganic phosphate in the deproteinized supernatant fractions, kept at 1 C, was determined immediately by the procedure of Fiske and SubbaRow (1925). Data were expressed as micrograms of inorganic phosphorus released per minute per milligram of protein during the period that zero-order kinetics were graphically observed. Preliminary experiments with *M. gallisepticum* A5969 demonstrated, within the pH range of 6.0 to 10.0, one optimum at pH 8.0; Mg⁺⁺, but not Ca⁺⁺, was essential for activity. These conditions were arbitrarily chosen for testing adenosine triphosphatase activity in all other *Mycoplasma* strains.

Glucose-6-phosphate dehydrogenase was estimated spectrophotometrically by following the increase in absorbance at 340 m μ upon addition of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) at 34 C. The reaction mixture (3.0 ml) contained 0.059 to 2.612 mg of cell protein (dictated by activity), and the following (in micromoles): NaCl, 23.4; Tris-HCl (pH 7.4), 7.5; 2-mercaptoethanol, 1.5; NAD or NADP, 7.2. The reaction was started with 20 μmoles of glucose-6-phosphate. Data were expressed as the increase in absorbance at 340 m μ per minute per milligram of protein.

Ribonuclease and deoxyribonuclease activities were estimated as described by Razin, Knyszynski, and Lifshitz (1964) by following the release of material absorbing at 260 m μ from reaction mixtures containing highly polymerized yeast ribonucleic acid (RNA) or highly polymerized sperm deoxyribonucleic acid (DNA), both from Calbiochem. The conditions for testing these enzymatic activities were those found optimal for nucleases of *M. laidlawii* (Razin et al., 1964). Data were expressed as the increase in absorbance at 260 m μ per hour per milligram of protein.

Protein was determined according to Lowry et al. (1951) by use of Pro-Sol (standardized protein solution, Standard Scientific Supply Corp., New York, N.Y.) as a standard.

Spectrophotometric assays were conducted in a Beckman DB spectrophotometer equipped with a model 14 Varicord recorder (Photovolt Corp., New York, N.Y.).

RESULTS

Enzymatic activities of cells and cell fractions. Table 1 shows that NADH₂ oxidase activity was associated with the membrane fraction of the

TABLE 1. *Enzymatic activities of Mycoplasma cells and cell fractions**

Organism	NADH ₂ oxidase			NADPH ₂ oxidase			Adenosine triphosphatase		
	W†	M†	S†	W	M	S	W	M	S
<i>M. laidlawii</i> A.....	0.20	4.92	1.20	0.00	0.00	0.00	0.70	9.02	0.00
<i>M. laidlawii</i> B.....	0.09	4.62	0.05	0.00	0.00	0.00	0.36	7.13	0.34
<i>M. gallisepticum</i> A5969.....	0.06	0.45	11.95	0.02	0.00	0.14	0.46	13.90	0.13
<i>M. gallisepticum</i> S6.....	0.03	0.48	14.11	0.00	0.00	0.12	0.08	0.97	0.00
<i>Mycoplasma</i> sp. 14.....	0.10	0.09	8.62	0.00	0.00	0.13	0.60	5.04	0.57
<i>M. neurolyticum</i>	0.01	0.62	2.43	0.00	0.00	0.37	0.14	1.29	0.00
<i>Mycoplasma</i> sp. H39.....	0.00	0.00	0.17	0.00	0.00	0.07	0.02	0.76	0.00
<i>M. bovis genitalium</i>	0.00	0.00	6.66	0.00	0.00	2.27	0.00	7.22	0.48
<i>M. hominis</i> ATCC 14152.....	0.02	0.00	0.07	0.00	0.00	0.01	0.25	1.77	0.00
<i>M. arthritis</i> ATCC 14124.....	0.00	0.00	0.06	0.00	0.00	0.01	0.00	0.37	0.00

* Data are expressed for NADH₂ and NADPH₂ as the decrease in absorbance at 340 mμ per minute per milligram of protein, and for adenosine triphosphatase as micrograms of inorganic phosphorus released per minute per milligram of protein; the data represent the mean value of at least three determinations with different batches of cells.

† Abbreviations: W, whole cells; M, membrane fraction; S, soluble fraction.

saprophytic *M. laidlawii* strains, whereas, in all other strains, NADH₂ oxidase activity was concentrated in the soluble fraction. NADH₂ oxidase activity in *Micrococcus lysodeikticus* protoplasts was confined entirely to the membrane. NADPH₂ oxidase activity was detected only in the soluble fraction of the parasitic strains. Adenosine triphosphatase activity was associated with the membrane fraction in all organisms. Glucose-6-phosphate dehydrogenase could only be demonstrated in the soluble fraction of *M. laidlawii* strains. The specific activities for *M. laidlawii* A and B were 0.23 and 0.18, respectively. NAD could not substitute for NADP in this reaction. Ribonuclease activity was usually found in both membrane and soluble fractions of *Mycoplasma* cells (Table 2), but was generally higher in the membrane fraction. In human and bovine *Mycoplasma* strains, deoxyribonuclease activity could not be demonstrated in the soluble fraction (Table 2). In the remaining strains, deoxyribonuclease activity was higher in the soluble than in the membrane fraction.

Effect of growth medium on enzyme localization. Experiments carried out with *M. laidlawii* B and *M. gallisepticum* A5969 showed that the location of the NADH₂ oxidase, NADPH₂ oxidase, glucose-6-phosphate dehydrogenase, and adenosine triphosphatase activities was not altered when cells were grown in Edward broth with 2% (v/v) PPLO Serum Fraction or swine serum, or in Tryptose broth with 1% of either serum. Penicillin and thallium acetate, included in Edward medium, were likewise without effect on the localization of the enzymes.

Various levels of PPLO Serum Fraction in

TABLE 2. *Nuclease activity of Mycoplasma cell fractions**

Organism	Ribo-nuclease		Deoxyribo-nuclease	
	M†	S†	M	S
<i>M. laidlawii</i> A.....	1.4	1.4	0.3	2.3
<i>M. laidlawii</i> B.....	1.2	0.4	0.7	2.9
<i>M. gallisepticum</i> A5969.....	0.3	0.0	0.1	0.3
<i>M. gallisepticum</i> S6.....	0.3	0.2	0.1	0.3
<i>Mycoplasma</i> sp. 14.....	0.2	0.4	0.0	0.8
<i>M. neurolyticum</i>	0.4	0.3	0.5	1.0
<i>Mycoplasma</i> sp. H39.....	1.2	0.3	0.4	0.0
<i>M. bovis genitalium</i>	8.5	0.5	0.4	0.1
<i>M. hominis</i> ATCC 14152.....	2.2	0.3	1.3	0.0
<i>M. arthritis</i> ATCC 14124.....	1.6	0.0	0.5	0.0

* Data are expressed as the increase in absorbance at 260 mμ per hour per milligram of protein and represent the mean value of at least three determinations with different batches of cells.

† Abbreviations: M, membrane fraction; S, soluble fraction.

Tryptose broth also did not alter the location pattern of enzymatic activities in *M. laidlawii* B. However, the specific activities were influenced to different degrees by the amount of PPLO Serum Fraction in the medium (Fig. 1).

Membrane-bound NADH₂ oxidase and adenosine triphosphatase in M. laidlawii B. To confirm the intimate association of NADH₂ oxidase and adenosine triphosphatase activities with the membranes of *M. laidlawii* B, a suspension of washed membranes was treated (Munro, Jackson, and Korner, 1964) with varying amounts of sodium

deoxycholate (NaDOC, Mann Research Laboratories, New York, N.Y.), and the enzymatic activity was determined. There was a decrease in absorbance at 400 m μ of the membrane suspension as the level of NaDOC increased (Fig. 2), indicating membrane dissolution. In the same samples, NADH₂ oxidase and adenosine triphos-

FIG. 1. Effect of PPLO Serum Fraction content in the growth medium on enzymatic activity of *Mycoplasma laidlawii* B cell fractions. Cells were grown for 24 hr at 37°C in Tryptose medium containing the indicated concentrations of serum fraction. The cells were lysed, fractionated, and tested for activity as described in Materials and Methods. Symbols: NADH₂ oxidase in membrane, ○; NADH₂ oxidase in soluble fraction, △; adenosine triphosphatase in membrane, □; glucose-6-phosphate dehydrogenase in soluble fraction, ●.

TABLE 3. Effect of deoxycholate treatment on the enzymatic activity of the washed ribosomal fraction of *Mycoplasma laidlawii* B*

Ribosomal fraction	NADH ₂ oxidase	Adenosine triphosphatase
Untreated	0.03	4.40
Treated†	0.00	0.00

* Ribosomes were washed and suspended in 1:20 "B2" buffer. Data are expressed as described in Table 1.

† The ribosomal fraction (500 μ g of protein per ml) was treated with 5 μ moles/ml of sodium deoxycholate for 10 min at room temperature and centrifuged at 105,000 $\times$ g for 2 hr. The residue was examined for activity.

FIG. 2. Dissolution of *Mycoplasma laidlawii* B membranes by sodium deoxycholate and its effect on enzymatic activities of the membranes. Membrane suspensions (containing 450 μ g of protein) were made up in 0.05 M Tris-HCl buffer, pH 8.2, and sodium deoxycholate was added to give a final volume of 3.0 ml. The suspensions were incubated for 10 min at room temperature. Enzymatic activities and absorbance at 400 m μ of the treated suspensions were determined as described in Materials and Methods. Symbols: absorbance, ○; NADH₂ oxidase, □; adenosine triphosphatase, △.

phatase activities increased as the membranes dissolved. At higher levels of NaDOC (above 4 to 6 μ moles/ml), both activities were reduced.

NADH₂ oxidase and adenosine triphosphatase activities in the ribosomal fraction. The ribosomal fraction isolated from *M. laidlawii* B showed low NADH₂ oxidase and adenosine triphosphatase activities. To test whether RNA material, presumably ribosomes, known to contaminate membrane fractions (Razin, Argaman, and Avigan, 1963) contributes to the enzymatic activities of membranes, the following experiment was conducted. A ribosomal fraction of *M. laidlawii* B was washed with 1:20 "B2" buffer, and was treated with NaDOC to dissolve contaminating membrane fragments. The treated ribosomal fraction lost all detectable NADH₂ oxidase and adenosine triphosphatase activities (Table 3).

DISCUSSION

Examination of cell fractions after disruption by various physical techniques (Pollack et al., 1965) demonstrated that osmotic lysis and freeze-thaw achieved the best separation of the soluble

and ribosomal fractions from membrane components in two *Mycoplasma* strains. Membranes of *M. laidlawii* isolated by use of osmotic lysis contained only trace amounts of nucleic acids (less than 1% of the total amount found in whole cells). Electron microscopy of these membrane preparations did not show any gross contamination with ribosomes or other cytoplasmic constituents (S. Razin, H. J. Morowitz, and T. Terry, *unpublished data*).

The location of the selected enzymes in *Mycoplasma* species resembles, with one exception, that found in other bacteria. Adenosine triphosphatase activity, confined to *Mycoplasma* membranes, usually has been localized in membrane fractions of other bacteria (Weibull, Greenawalt, and Löw, 1962; Hughes, 1962; Drapeau and MacLeod, 1963). Localization of adenosine triphosphatase in the membrane region of *M. gallisepticum* A5969 was recently accomplished by use of cytochemical techniques with thin sections and electron microscopy (J. Maniloff, H. J. Morowitz, R. J. Barnett, and M. Munkres, *unpublished data*). Glucose-6-phosphate dehydrogenase, found exclusively in the soluble fraction of *M. laidlawii* A and B, was also found in the soluble fraction of *Pseudomonas fluorescens* (Burrous and Wood, 1962) and *P. aeruginosa* (Campbell, Hogg, and Strasdine, 1962). However, the location of NADH₂ oxidase in the parasitic *Mycoplasma* species differs from that of most other bacteria. In the parasitic *Mycoplasma* species, this activity was found predominantly in the soluble fraction, whereas in other bacteria it has been generally confined to the membrane fraction (Weibull, Beckman, and Bergström, 1959; Marr, 1960; Robrish and Marr, 1962; Hughes, 1962; Burrous and Wood, 1962; Jones and King, 1964; McDonald, 1964; White and Smith, 1964). Soluble NADH₂ oxidase activity has been demonstrated, however, in extracts of sporogenous bacteria (Dolin, 1959a, b; Doi and Halvorson, 1961a, b; Dworkin and Niederpruem, 1964; Pepper and Costilow, 1965). Yet, the finding of NADH₂ oxidase activity in the soluble fraction may represent in certain cases dissolution of particles during the drastic disruption procedures. Thus, Downey (1964) showed that membrane-bound NADH₂ oxidase of *Bacillus subtilis* protoplasts was released into a soluble fraction after sonic treatment. Campbell et al. (1962) subjected *P. aeruginosa* spheroplasts to a brief sonic treatment and found NADH₂ oxidase activity in a "cytoplasm" fraction. However, after centrifugation at 100,000 × *g* for 2 hr, all the "cytoplasm" activity was sedimented.

The saprophytic *M. laidlawii* A and B resembled most other bacteria in the location of

NADH₂ oxidase activity in the membrane. This is another important difference separating *M. laidlawii* from all other *Mycoplasma* species. These differences include the ability of *M. laidlawii* to synthesize carotenoids (Rothblat and Smith, 1961), its independence of cholesterol for growth (Razin and Knight, 1960), and a high osmotic fragility (Razin, 1963, 1964). Such features indicate a difference in membrane composition existing between *M. laidlawii* and other *Mycoplasma* species.

The inability to demonstrate glucose-6-phosphate dehydrogenase activity in the soluble fractions of all parasitic *Mycoplasma* species tested may be due to the presence of NADH₂ and NADPH₂ oxidase activities in these fractions. The techniques employed followed the reduction of NAD or NADP spectrophotometrically, and the presence of such oxidases could make glucose-6-phosphate dehydrogenase activity undetectable.

The association of NADH₂ oxidase and adenosine triphosphatase with *M. laidlawii* membrane architecture was demonstrated by the marked increase in activity of these enzymes upon dissolution of the membrane by sodium deoxycholate. Dissolution of the membrane apparently exposed active enzyme sites. NADH₂ oxidase was shown to form an integral part of the membrane subunits of *M. laidlawii* B (S. Razin, H. J. Morowitz, and T. Terry, *unpublished data*). At higher concentrations of the detergent, the decrease in activity is assumed to be due to denaturation of enzyme proteins.

The higher activities observed in *Mycoplasma* membrane fractions when compared to whole cells (Table 1) may be accounted for, at least in part, by the exposure of more enzyme sites in these isolated membranes and by the low cellular permeability of substrates like NADH₂ or ATP (Mitchell, 1962; McDonald, 1964).

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