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Differences in Incorporation of Nucleic Acid Bases and Nucleosides by Various *Mycoplasma* and *Acholeplasma* Species

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Eight species representative of the serological diversity of the *Mycoplasmatales* were tested for their ability to incorporate radiolabeled nucleic acid precursors into acid-insoluble material. Cultures in complex growth medium were centrifuged and resuspended in minimal essential medium (Eagle). For *Acholeplasma laidlawii*, labeling occurred mainly during the first 4 h of incubation, with substrate saturation at 20 μM. All organisms tested incorporated uracil, adenine, and guanine; none incorporated cytosine. Thymine was incorporated only by bovine group 7, *Mycoplasma putrefaciens*, and *Mycoplasma pneumoniae* (strain 3546), but deoxynucleosides enhanced thymine incorporation in *A. laidlawii*, *Mycoplasma gallisepticum*, *M. pneumoniae* (strain AP-164), and *Mycoplasma hyorhinis*. Nucleoside incorporation (adenosine, guanosine, uridine, cytidine, and thymidine) was not observed for the arginine-utilizing species, *Mycoplasma hominis* and *Mycoplasma arginini*, whereas all other organisms tested incorporated nucleosides. The incorporation pattern provides additional metabolic evidence to support the biochemical and antigenic diversity of these organisms. The recognition of differences in incorporation of nucleic acid precursors is important not only to the specific labeling of these organisms, but also to the study of metabolism and transport.

The organisms in the *Mycoplasmatales* are unusual in that they have a nutritional requirement for nucleic acids or precursors (7, 25, 36, 41), suggesting that they are defective in synthesis of nucleic acid precursors. Nucleic acid precursors in partially or completely defined media have included both purine and pyrimidine bases and/or nucleosides (38, 42, 46). However, the specific requirements have been defined for only a few easily grown species; additionally, our ability to generalize on their precursor requirements is complicated by the biological diversity of the order (1, 27). Their heterogeneity was initially recognized by the separation of family *Acholeplasmataceae* from *Mycoplasmataceae* (8); but the complexity of the organisms still classified in genus *Mycoplasma* is such that this genus appears to contain at least five groups of quite distinct organisms based on antigenic comparisons (17, 19, 21), a division which parallels their genetic and metabolic diversity (1, 27).

The present study is focused on determining the ability of *Acholeplasma laidlawii* and seven *Mycoplasma* species (representative of 5 serological groups) to incorporate free bases and nucleosides into acid-insoluble material. We found that significant incorporation could not be obtained in complex media, likely because of the competition provided by nucleic acid breakdown products of the yeast extract component of the medium, and that incorporation was unsatisfactory in cells suspended in saline. However, both modest growth and DNA labeling have been obtained in Eagle basal medium supplemented with serum (35, 42), and uptake of uracil into mycoplasmata has been demonstrated in contaminated cell cultures in Eagle minimum essential medium in the absence of serum (20). The omission of serum enhanced uptake of uracil because serum contains uracil or uracil precursors (20, 31). Accordingly, the incorporation studies were carried out in minimum essential medium without serum using organisms concentrated from undefined medium.

MATERIALS AND METHODS

Organisms. *Mycoplasma* and *Acholeplasma* sp. were obtained from the American Type Culture Collection or from individual sources: *Mycoplasma* sp. bovine group 7 (strain N-29B, J. Tully; 5), *Mycoplasma putrefaciens* ATCC 15718 (47), *A. laidlawii* ATCC 14192, *Mycoplasma gallisepticum* ATCC

15302, *Mycoplasma pneumoniae* AP-164 (this laboratory, passage 16 to 20 from patient; 22), *M. pneumoniae* 3546 (this laboratory, passage 125 to 130 from patient, a non-spherule-forming strain), *Mycoplasma hyorhinis* ATCC 25021, *Mycoplasma arginini* (strain G-230, obtained from M. F. Barile), and *Mycoplasma hominis* ATCC 14027. The organisms were selected as being representative of the spectrum of antigenic differences and similarities in the *Mycoplasmatales* (19, 21).

Media. Soy peptone fresh yeast "dialysate broth" medium (16) was used for the growth of the organisms. The broth base was supplemented with 10% "agamma" horse serum (Flow Laboratories; the gamma globulin content was reduced by fractionation from whole horse serum), 10 mM *N*-tris(hydroxmethyl)methyl-2-aminoethane-sulfonic acid (Calbiochem), and 100 U of penicillin per ml at a final pH of 7.3. Colony-forming units (CFU) were determined by plate count on soy peptone agar medium (18) supplemented with 25% whole horse serum, 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, and penicillin (100 U/ml) at a final pH of 7.4. Eagle minimal essential medium (6) was prepared in Hanks balanced salt solution (11), with 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid and 100 U of penicillin per ml at a final pH of 7.3.

Nucleic acid precursors. The following tritiated compounds (with specific activities) were obtained from Schwarz/Mann: [6-^{3}H]uracil, 24 Ci/mmol; [*methyl*-^{3}H]thymine, 14 Ci/mmol; [8-^{3}H]adenine, 10 Ci/mmol; [8-^{3}H]guanine, 5.5 Ci/mmol; [5-^{3}H]cytosine, 21 Ci/mmol; [6-^{3}H]uridine, 23 Ci/mmol; [*methyl*-^{3}H]-thymidine, 15 Ci/mmol; [2-^{3}H]adenosine, 16 Ci/mmol; [8-^{3}H]guanosine, 6.6 Ci/mmol; [5-^{3}H]cytidine, 19 Ci/mmol. [2-^{14}C]thymine and [2-^{14}C]cytosine, specific activity 61 mCi/mmol for each, were obtained from Amersham/Searle. Unlabeled nucleic acid bases and nucleosides were obtained from Calbiochem, but uracil and adenine were obtained from the Sigma Chemical Co.

Incorporation assay. Broth cultures were grown at 37°C to the first sign of a visible haze. For some experiments, cultures were diluted 1:10 in warm growth medium and incubated for 3 h prior to harvesting, to obtain exponentially growing cells. The cells were centrifuged at 13,000 × *g* for 30 min and resuspended in minimum essential medium to a concentration 100 times that of the original broth culture. Midexponential-phase organisms were utilized for all experiments, and cell suspensions were adjusted to approximately 10^9 CFU/ml (for actual CFU values see Tables 1 and 2). These precautions were necessary because uracil and adenine incorporation in *A. laidlawii* was maximal in log phase, depressed in stationary phase, and only slightly inhibited at concentrations of cells in excess of 10^{10} CFU/ml. In contrast, incorporation was strikingly sensitive to cell density in *M. arginini* (inhibition was marked beyond 10^9 CFU/ml) but less sensitive to culture age than in *A. laidlawii*. The cell suspension (25 μl) was added to individual wells of a microtiter tray. Test radiochemical solution (diluted in minimum essential medium with 2 μM unlabeled test compound unless otherwise indicated) was added to each well in a 25-μl volume. Experiments were carried out in triplicate. The tray was incubated for 2 h (or indicated time periods) in a 37°C water bath, after which each well received 1 drop (approximately 25 μl) of 2 mg of bovine albumin per ml (to serve as carrier) and trichloroacetic acid to a final concentration of 25% (wt/vol). All additions were carried out on ice. After 60 min, precipitates were filtered onto Whatman GF/A or GF/C glass fiber filter paper by means of an automatic sample harvester (12) utilizing chilled washes of 5% trichloroacetic acid and then 100% methanol. The filtered samples were assayed for radioactivity in the tritium or carbon-14 range using Bray solution (3) and a Packard Tri-Carb scintillation counter at a counting efficiency of 52% for tritium and 84% for carbon-14.

Incorporation, expressed as millimoles per viable CFU in this study, is computed as follows: $a \times b/c \times d$, where a = total substrate available in millimoles, b = labeled substrate incorporated in counts per minute, c = labeled substrate available in counts per minute, and d = total CFU in test. The background for each individual experiment (using medium lacking organisms) was subtracted.

RESULTS

Determination of optimum labeling conditions. The optimal incorporation time was measured by mixing a suspension of *A. laidlawii* with various radiolabeled compounds and sampling at indicated times. Incorporation of uracil, adenine, and thymidine was linear for approximately 4 h; thereafter, no further incorporation was observed (Fig. 1). The counts per minute observed for thymidine and adenine were similar to those shown for uracil. Levels of incorporation were similar for uridine, adenosine, guanine, guanosine, and cytidine. Regardless of length of incubation, thymine and cytosine were not incorporated, nor was uracil incorporated into heat-killed cells (56°C for 30 min).

The effect of substrate concentration on incorporation by *A. laidlawii* was tested by using varying amounts of unlabeled precursor with the isotope. Incorporation of uracil, uridine, and adenine was saturated at 20 μM (Fig. 2), but incorporation of thymidine did not show saturation. Subsequent experiments were run with 2 μM substrate in addition to the small quantity (around 0.1 μM) defined by the specific activity of the labeled compound to maximize incorporation while maintaining significant labeling.

Differences in incorporation of nucleic acid bases and nucleosides in species in the *Mycoplasmatales*. The incorporation of five nucleic acid bases and five nucleosides was tested in suspensions of *A. laidlawii* and a variety of mycoplasmic species representative of the heterogeneity of genus *Mycoplasma* (1, 19, 21, 27). The results are presented relative to

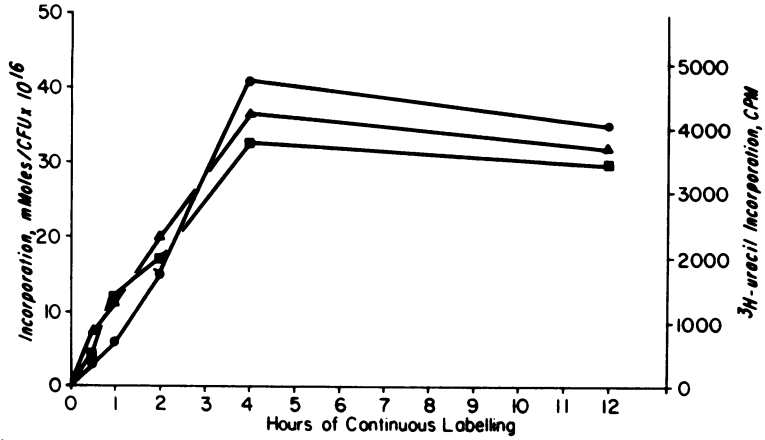

FIG. 1. *Continuous labeling of A. laidlawii. Individual cultures for each radiolabeled compound, containing 2.16 × 10⁷ CFU/ml, were sampled by taking 50-μl portions at various time points and assaying for incorporation as described in the text. Each sample contained the following initial amounts of radioactivity: uracil (●), 2.32 × 10⁵ cpm; adenine (▲), 1.11 × 10⁵ cpm; and thymidine (■), 1.46 × 10⁵ cpm.*

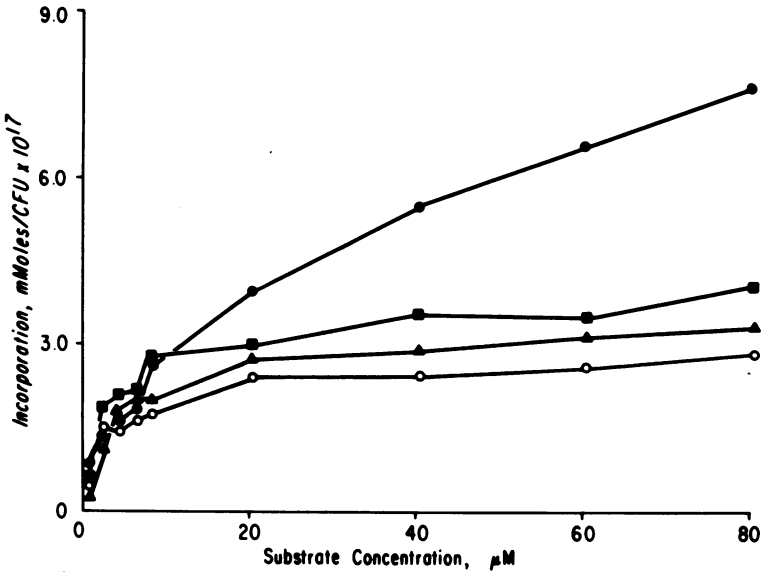

FIG. 2. *Incorporation versus substrate concentration in A. laidlawii. Each test, performed as described in the text, contained the following initial amounts of radioactivity and cells (CFU): uracil (▲), 1.20 × 10⁶ cpm, 1.81 × 10⁸ CFU; adenine (○), 5.91 × 10⁵ cpm, 2.86 × 10⁸ CFU; uridine (■), 5.86 × 10⁵ cpm, 3.32 × 10⁸ CFU; and thymidine (●), 6.89 × 10⁵ cpm, 1.95 × 10⁸ CFU.*

CFU to provide a comparative basis for different experiments or different parts of the same experiment. The CFU base was chosen because it was the most easily determined value; other values such as protein would have provided more quantitative data, but the small yield of log-phase cells (0.5 to 2 mg/liter) would have been overwhelmed by the relatively huge amount of horse serum in the medium (7,000 mg/liter). Coprecipitation of horse serum components with pelleted mycoplasmata is well recognized (9, 37, 48) and provides a significant obstacle to research with mycoplasmata. Unfortunately, defined media that provide good yields are not available presently. The use of CFU as a base, however, also causes problems. The organisms may grow in aggregated form to varying degrees, causing the CFU values to be substantially less than the actual number of organisms. Accordingly, incorporation values per CFU will

be inflated for organisms which grow as aggregates or which have a low plating efficiency. Aggregation would be the greatest problem for *M. pneumoniae* AP-164 (2) and a lesser problem (but variable) with the other species tested. Therefore, the comparability of these data between organisms has certain difficulties. However, the values shown in Table 1 for any one organism were taken from a single experiment, and experiments were carried out under identical conditions.

Since adenine was incorporated relatively well by all organisms tested, the incorporation values for this compound were chosen to serve as the denominator for the determination of an incorporation fraction for each compound for each organism, the incorporation value for the compound in question serving as the numerator. Variations in the characteristics of experimental material, such as cell concentration, degree of cell aggregation, and metabolic activity, are thus accounted for. The fractions denoting incorporation relative to adenine are therefore presented as characteristics of any organism (Table 1). Four tests of this approach with *A. laidlawii* gave reproducible results (not shown).

Adenine, guanine, and uracil were incorporated by all organisms tested, although lower values were obtained for guanine and uracil in *M. pneumoniae* AP-164 and for guanine in *M. gallisepticum* and *M. pneumoniae* 3546. Cytosine was not incorporated by any organism. The most striking difference in incorporation among the free bases was the fact that *M. pneumoniae* 3546 was the only organism outside of the serological group I organisms (bovine group 7 and *M. putrefaciens*) which incorporated thymine at a level near that of adenine. Slight incorporation was observed in *M. gallisepticum* and *M. pneumoniae* AP-164. The fact that cytosine was not incorporated by any organism and thymine only by certain species could have been the result of dehydrogenation of the 5-^{3}H label of cytosine or demethylation of the 5-*methyl*-^{3}H label of thymine. However, [2-^{14}C]cytosine was not incorporated by any species, and only those organisms that incorporated [*methyl*-^{3}H]thymine were able to incorporate [2-^{14}C]thymine (data not shown). Another striking difference was the fact that the arginine-utilizing organisms (*M. hominis* and *M. arginini*; serological group VII) did not incorporate nucleosides, aside from small amounts of guanosine, whereas the other organisms incorporated all nucleosides. *M. arginini* did not incorporate thymidine even when tested in a mixture of adenosine, guanosine, and cytidine (25 μM each) or in 25 μM deoxyadenosine. Similarly, uridine was not incorporated in the presence of a mixture of adenosine, thymidine, guanosine, and cytidine (25 μM each) or in 25 μM thymidine. Additionally, *M. arginini* did not incorporate thymidine or uridine when cell density and age were varied.

The effect of ribonucleosides and deoxyribonucleosides on thymine incorporation. Thymine uptake occurs in the presence of deoxyribosyl donors in *Escherichia coli* and *Haemophilus influenzae*, organisms which normally

TABLE 1. *Incorporation of free bases and nucleosides into various Mycoplasma and Acholeplasma species*[a]

Species	CFU/ml (×10^{6})	Serological group[b]	Adenine incorporation (mmol/CFU × 10^{16})	Relative incorporation[c] (as a fraction of adenine incorporation)								
				G	U	C	T	AR	GR	UR	CR	TdR
M. putrefaciens ATCC 15718	290	I	5.22	3.81	1.19	0.0213	4.37	1.65	3.54	2.26	0.914	4.87
Bovine group 7 (N29-B)	100	I	21.7	1.08	0.544	0.0118	0.567	1.44	1.02	0.912	0.283	0.613
A. laidlawii ATCC 14192	10.8	II	20.1	1.05	0.766	0.0111	0.0224	1.39	1.10	1.72	0.297	0.851
M. gallisepticum ATCC 15302	129	IV	1.07	0.289	1.22	0.0280	0.131	3.43	4.10	5.41	1.61	2.21
M. pneumoniae[d] AP-164	23.4	V	10.51	0.141	0.271	0.00742	0.181	0.717	2.04	1.17	0.817	2.76
M. pneumoniae 3546	92	V	0.265	0.237	0.608	0.0166	0.687	4.34	5.85	4.53	6.57	1.91
M. hyorhinis ATCC 25021	2,900	VI	0.380	1.36	1.25	0.0153	0.0721	2.05	1.92	2.92	1.58	0.416
M. arginini G-230	448	VII	0.398	2.81	1.70	0.0540	0[e]	0.0510	0.122	0.0568	0.0148	0.0575
M. hominis ATCC 14027	360	VII	0.773	0.545	1.11	0	0	0.0944	0.136	0.0893	0	0.0712

[a] Tests were performed as described in the text and contained 0.5 × 10^{5} to 2.0 × 10^{5} cpm of radioactivity.

[b] Ref. 19 and 21.

[c] Abbreviations: A, adenine; G, guanine; U, uracil; C, cytosine; T, thymine; R, riboside; dR, deoxyriboside. Incorporation is expressed as a fraction relative to adenine incorporation for that same organism: incorporation of compound in question (mmol/CFU × 10^{16})/incorporation of adenine (mmol/CFU × 10^{16}).

[d] See text for discussion of reliability of *M. pneumoniae* AP-164 CFU values.

[e] 0, Experimental value below background.

do not utilize exogenous thymine (4, 14, 15). Similarly, we found that deoxynucleosides (excluding thymidine) markedly enhanced thymine incorporation for *A. laidlawii*, *M. pneumoniae* AP-164, *M. hyorhinis*, and *M. gallisepticum* (Table 2). Moderate enhancement occurred with pyrimidine deoxynucleosides for bovine group 7 and with deoxyguanosine for *M. pneumoniae* 3546. Ribonucleosides, however, did not enhance thymine incorporation when three species were tested (*A. laidlawii*, *M. hyorhinis*, and *M. pneumoniae* AP-164; data not shown). Thymine incorporation was minimal or undetectable for *M. arginini* and *M. hominis* even in the presence of deoxynucleosides.

DISCUSSION

Thymine utilization in the absence of deoxyribosyl donors by organisms related to *M. mycoides* (i.e., bovine group 7 and *M. putrefaciens*, Tables 1 and 2) is not surprising since a growth requirement for thymine was found in formulating a defined medium for *Mycoplasma* strain Y, another *M. mycoides*-type organism (38). As in *E. coli* (15) and *H. influenzae* (4), *A. laidlawii*, *M. gallisepticum*, *M. pneumoniae* AP-164, and *M. hyorhinis* incorporate thymine poorly unless exogenously provided with a deoxyribosyl donor (as deoxynucleosides, Table 2). The differences found in thymine incorporation between the two strains of *M. pneumoniae* may result from the fact that strain 3546 is a high-passage strain which grows more rapidly than strain AP-164 and which produces few spherules (Kenny, unpublished data).

The failure of any organism to incorporate cytosine is in striking contrast to their ability to incorporate cytidine. However, Mitchell and Finch (28) recently have shown that cytidine is deaminated to uridine in cultures of *Mycoplasma mycoides*.

The uptake of adenine, guanine, and uracil (Table 1) could be initiated by phosphoribosyltransferases, which have been demonstrated for adenine in *M. mycoides* (40), hypoxanthine in *A. laidlawii* and *M. hyorhinis* (43), and uracil in a number of species (26). However, nucleoside phosphorylase activities are also known for these organisms (adenosine phosphorylase [13] and uridine phosphorylase, EC 2.4.2.3 [24]).

The failure of the arginine-utilizing species *M. hominis* and *M. arginini* to incorporate nucleosides cannot be explained on the basis of their lack of appropriate metabolic machinery, since these organisms have nucleoside phosphorylase (13, 24) and thymidine kinase activity has been demonstrated in *M. hominis* (44). The alternative explanation is that the organisms are impermeable to nucleosides (as are some mutants of *E. coli*; 23, 29), a conclusion which is supported by our recent experiments. Although *M. arginini* accumulates an intracellular pool of adenine (not acid precipitable) during short incubation periods (5 min), adenosine was not taken up. This is in contrast to the uptake of both compounds by *A. laidlawii* (McIvor and Kenny, unpublished data). This impermeability is further borne out by the fact that whereas cell lysates of *M. arginini* can cleave uridine to uracil, this activity was not observed in whole cell preparations grown as described herein (McIvor and Kenny, unpublished data). Thus, the specific DNA labeling of these organisms poses a problem since they incorporate neither thymine nor thymidine.

The incorporation of nucleosides into species

TABLE 2. *Effect of deoxyribonucleosides on thymine incorporation for various species of Mycoplasma and Acholeplasma*[a]

Species	CFU/ml ($\times 10^6$)	Serological group[b]	Adenine incorporation[c] (mmol/CFU $\times 10^{16}$)	Thymine incorporation (mmol/CFU $\times 10^{16}$) with additives[d]: None	AdR	GdR	UdR	CdR	TdR
M. putrefaciens	693	I	2.74	4.97	5.54	4.23	3.89	3.96	0.0521
Bovine group 7	360	I	2.12	7.38	6.35	6.93	12.72	10.69	0.970
A. laidlawii	1,110	II	0.689	0.0129	0.136	0.358	0.0643	0.0272	0.00396
M. gallisepticum	21.1	IV	13.23	0.730	5.73	3.81	7.55	7.38	0
M. pneumoniae AP-164[e]	9	V	14.20	3.11	10.35	10.02	6.06	6.74	2.24
M. pneumoniae 3546	1,970	V	1.42	1.27	1.75	2.61	0.969	1.28	0.0985
M. hyorhinis	229	VI	0.956	0.185	0.662	0.777	1.75	1.39	0.0225
M. arginini	55	VII	1.97	0[f]	0.0124	0.0186	0.0295	0	0
M. hominis	51	VII	2.47	0	0.127	0.0134	0.101	0.0138	0

[a] Tests were performed as described in the text and contained a final volume of 75 μl.
[b] Ref. 19 and 21.
[c] Provided as a positive control.
[d] Abbreviations are as in Table 1. Deoxyribonucleosides were at a final concentration of 1.0 mM.
[e] See text for discussion of reliability of CFU values for *M. pneumoniae* AP-164.
[f] 0, Experimental value below background.

other than the arginine utilizers might be complicated by the presence of nucleoside phosphorylase activities. Poor incorporation would result for those nucleosides that are cleaved to bases which are inefficiently utilized by the organisms if the rate of nucleoside phosphorylase activity substantially exceeded the rate of incorporation. In cell culture, nucleoside phosphorylase activity is rapid (10, 33), and cleavage prevents the incorporation of uridine or thymidine into animal cells (which do not incorporate their free bases). This could affect the incorporation of cytidine, since cytosine was not incorporated by any organism. Four organisms (bovine group 7, *A. laidlawii*, *M. gallisepticum*, and *M. hyorhinis*, Table 1) showed lower incorporation of cytidine than adenosine. Similarly, incorporation of thymidine would also be affected by those organisms that do not incorporate thymine; this effect was pronounced for *M. hyorhinis*.

The incorporation of nucleic acid precursors, most notably that of thymidine (30, 45) and uracil (20, 32, 39), has been utilized in detecting the presence of mycoplasmata in cell cultures. According to the results presented here, uracil would be the optimum differential label for detecting the contamination of cell cultures by any one of the species tested, as free purine bases are readily incorporated into animal cells (34). The arginine-utilizing species apparently would not be detected by the method of thymidine labeling followed by autoradiography and microscopic examination for silver grains over the cytoplasm of the culture cell.

These results resolve the tested organisms into three groups on the basis of their thymine incorporation behavior: (i) glycolytic species, which incorporate thymine in the absence of a deoxynucleoside supplement (bovine group 7, *M. putrefaciens*, and *M. pneumoniae* 3546); (ii) glycolytic organisms, which incorporate thymine in the presence of deoxynucleosides but poorly in the absence of supplements (*A. laidlawii*, *M. gallisepticum*, *M. pneumoniae* AP-164, and *M. hyorhinis*); and (iii) the arginine-utilizing species, which do not incorporate thymine under any condition tested. More significantly, this latter group (*M. arginini* and *M. hominis*) does not incorporate nucleosides at all, in contrast to the other two groups. The incorporation pattern (Table 1) lends support to the idea of dividing mycoplasmic species into groups serologically (21). Not only are *M. putrefaciens* and bovine group 7 related to *M. mycoides* serologically (21), but also the metabolic similarities in thymine incorporation reported herein suggest a biochemical relationship. The close immunological relatedness among the arginine-utilizing organisms (21) is paralleled by the lack of nucleoside incorporation in both *M. arginini* and *M. hominis*. No outstanding differences in incorporation were found among *A. laidlawii*, *M. gallisepticum*, *M. pneumoniae* AP-164, and *M. hyorhinis*, even though these species are strikingly serologically heterogeneous and are separated into four different groups. The results presented here clearly amplify the biochemical differences among the serotaxonomic groups within the *Mycoplasmatales*.

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