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Adenine Formation from Adenosine by Mycoplasmas: Adenosine Phosphorylase Activity

(purine-nucleoside phosphorylase/enzymic phosphorylation of adenosine/screening of mycoplasma contamination)

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ABSTRACT **Mammalian cells have enzymes to convert adenosine to inosine by deamination and inosine to hypoxanthine by phosphorolysis, but they do not possess the enzymes necessary to form the free base, adenine, from adenosine. Mycoplasmas grown in broth or in cell cultures can produce adenine from adenosine. This activity was detected in a variety of mycoplasmatales, and the enzyme was shown to be adenosine phosphorylase. Adenosine formation from adenine and ribose 1-phosphate, the reverse reaction of adenine formation from adenosine, was also observed with the mycoplasma enzyme.**

Adenosine phosphorylase is apparently common to the mycoplasmatales but it is not universal, and the organisms can be divided into three groups on the basis of their use of adenosine as substrate. Thirteen of 16 *Mycoplasma*, *Acholeplasma*, and *Spiroplasma* species tested exhibit adenosine phosphorylase activity. *M. lipophilium* differed from the other mycoplasmas and shared with mammalian cells the ability to convert adenosine to inosine by deamination. *M. pneumoniae* and the unclassified *M. sp.* 70-159 showed no reaction with adenosine. Adenosine phosphorylase activity offers an additional method for the detection of mycoplasma contamination of cells. The patterns of nucleoside metabolism will provide additional characteristics for identification of mycoplasmas and also may provide new insight into the classification of mycoplasmas.

In 1945, Kalckar (1) discovered purine-nucleoside phosphorylase (EC 2.4.2.1; purine-nucleoside:orthophosphate ribosyltransferase), which catalyzes the phosphorolysis of the nucleosides of hypoxanthine and guanine. Later, Friedkin and Kalckar (2) demonstrated that adenosine was not a substrate for the enzyme isolated from eukaryotic cells. In the case of prokaryotes, Gardner and Kornberg (3) reported that the enzyme purified from *Bacillus cereus var. terminalis* catalyzed the phosphorolysis of the same purine nucleosides as observed by eukaryotic enzymes, but again adenosine was not catalyzed as a substrate. However, Munch-Petersen (4) reported that purine-nucleoside phosphorylase was induced when wild-type *Escherichia coli* was grown in the presence of any of the purine nucleosides, including adenosine. Similar findings were reported with *Salmonella typhimurium* (5). After these biological findings, Robertson and Hoffee (6) purified the purine-nucleoside phosphorylase from *S. typhimurium* extensively and demonstrated that adenosine could also be used as a substrate by this enzyme.

Mycoplasmas are the smallest free-living prokaryotic organisms (130–300 nm in diameter, 0.1–0.2 μm^3 in volume, with 5 to 10 $\times$ 10^8 daltons of DNA, and without a cell wall), and are notorious for frequent and silent contamination of animal cell cultures (7). To many investigators, mycoplasmas are regarded as a nuisance and are often ignored in experimental procedures since they often exert no obvious effect on the well-being of the cells. However, mycoplasmas do indeed alter the cells in terms of macromolecular synthesis, stability of genetic materials, and other parameters, causing misinterpretation of many biochemical and biological findings (7).

We found that most mycoplasmas possess the ability to produce adenine from adenosine, similar to certain bacteria. This activity was not found in any animal cell cultures tested, unless contaminated by mycoplasmas. Thus, one application of this finding is another method for detection of mycoplasma contamination in animal cell cultures.

MATERIALS AND METHODS

Mycoplasmatales. Twelve of the mycoplasma cultures used in this study were derived from authentic type strains (8); *Spiroplasma citri* strain R8-A8 was kindly provided by J. G. Tully. Mycoplasma strain 70-213 was isolated from a bovine lung and is related to a group of organisms that we have isolated from cell cultures and bovine serum. Recently, an antigenic relationship was found with 70-213 and the Donetta strain (M. F. Barile, personal communication). We therefore classify strain 70-213 as *M. agalactiae* var. *bovis*, although the status of this organism remains in question.

Mycoplasma strains related to strain 70-159 were first isolated in our laboratory in 1968. Since then we have isolated over 100 strains, all from cell cultures, which comprise a group distinct from the known mycoplasmas. To our knowledge, members of the 70-159 group have never been isolated from any source other than the cultured cell.

Mycoplasma Cultures. The agar medium used to isolate mycoplasmas from infected cell cultures and the liquid medium used to propagate mycoplasmas for enzyme assay have been described (8) and are referred to as modified solid medium (Flow no. 5-059) and modified liquid medium (Flow no. 5-056).

For the production of concentrated mycoplasma suspensions, 0.5 ml of frozen seed stock was inoculated into 500 ml of modified liquid medium. The cultures were incubated aerobically at 36°, with the exception of *S. citri*, which was incubated anaerobically at 32°. The mycoplasma cells were harvested when the medium changed 0.5–1.0 pH unit, as determined by inspection of the phenol red indicator. Previous growth curve studies revealed that the first visually detectable pH change (approximately 0.5 pH unit) coincided with the

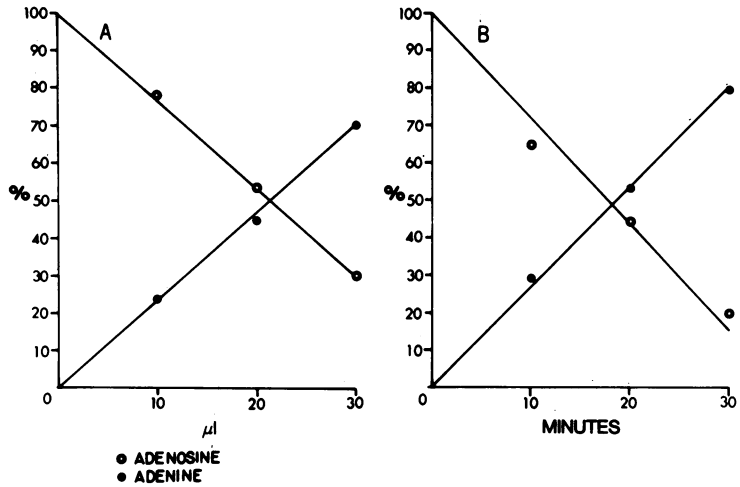

FIG. 1. Adenine formation from adenosine by HEP/6 MP cells infected with *M. hyorhinis*. (A) The assay mixture consists of 50 μl of 10 μM [2,8-^{3}H]adenosine, 50 μl of 10 mM phosphate buffer (pH 7.4). The infected cell extract was added in 10-, 20-, and 30-μl portions with enough water to make the final volume 200 μl. The cell extract had a protein concentration of 5–6 mg/ml. The reaction was stopped after 20 min of incubation at 37° by addition of 30 μl of 60% perchloric acid and 70 μl of 5 M KOH, and the mixture was kept standing in ice for 15 min. The precipitates were removed by centrifugation for 10 min at 3000 rpm by IEC centrifuge. (B) Twenty microliters of infected cell extract was used and incubated for 10, 20, and 30 min at 37°. Three microliters of the supernatant solution (total radioactivity, about 1200 cpm) were spotted on thin-layer plates by solvent no. 4. Spots of adenosine and adenine were eluted and radioactivity was determined as described in *Materials and Methods*.

maximal viability titer. Before concentration, the titers ranged from 10^6 to 10^9 colony-forming-units/ml.

Immunofluorescence Procedures were used for detection and identification of mycoplasmas infecting cell cultures and mycoplasma colonies growing on agar medium. Cell cultures were grown on glass coverslips contained in 35 mm plastic petri dishes. Coincident with the biochemical test, the coverslips were removed, washed with phosphate-buffered saline, and fixed with absolute ethanol. The fixed cells were then reacted for 30 min with a 1:100 dilution of the appropriate anti-mycoplasma fluorescein-conjugated globulin. Unfixed mycoplasma colonies growing on the surface of agar medium were reacted directly with conjugates and examined by use of incident UV excitation (9).

TABLE 1. *Requirement of phosphate for adenine formation from adenosine by a mycoplasma*

Source of the enzyme	Adenine formation			
	Control		With *M. hyorhinis*	
	Mouse cell	Human cell	Mouse cell	Human cell
[^{3}H]Adenosine				
+ phosphate	0*	0	65*	75
− phosphate	0	0	2	8

Cultured cells were disrupted by sonication at 4° for a total of 10 sec. The extracts were centrifuged for 40 min at 25,000 × *g*, and the supernatants were dialyzed against 10 mM Tris·HCl (pH 7.4) for 2 days at 7°. The assay mixture contained 50 μl of 10 mM phosphate (pH 7.4), 50 μl of 1 μM [^{3}H]adenosine, and 50 μl of cell dialysates, and was incubated at 37° for 20 min. The reaction was stopped by chilling in ice and adding 70 μl of 60% perchloric acid and 150 μl of 5 M KOH. After the mixture stood for 15 min in ice, the precipitates were removed by centrifugation at 3000 rpm for 10 min. Ten microliters of the supernates was spotted on thin-layer plates and developed by solvent buffer no. 4. Controls produced inosine and hypoxanthine with phosphate, and inosine without phosphate. Cells contaminated by *M. hyorhinis* produced mainly adenine, with 5–10% of hypoxanthine in the presence of phosphate, and inosine without phosphate. Cells used for this assay were described in *Materials and Methods*.

* The value indicates the percentage in the adenine spot of the total radioactivity recovered from thin-layer chromatography.

Enzyme Preparation from Mycoplasmas. Mycoplasma grown in 500 ml of broth were centrifuged for 90 min at 19,000 rpm in a no. 19 rotor of a Beckman ultracentrifuge. The precipitate was suspended in about 2.0 ml of 10 mM Tris·HCl buffer (pH 7.4), with 2 mM dithiothreitol per centrifuge tube, and sonicated three times for 5 sec (total volume, 1–5 ml). The disrupted extract was then centrifuged for 30 min at 15,000 rpm in a no. 19 rotor. The supernates used for assay usually contained 2–5 mg/ml of protein, which consisted primarily of serum proteins from the broth culture.

Animal Cell Cultures. In the practical application of detection of mycoplasma in cultured cells, mouse 3T6 (10), human HEP (11), and Graffi hamster cells (12) were used as control cultures; these cells were monitored for freedom from mycoplasma contamination throughout this study. Mouse MIRT (13) and human HEP/6MP (13), chronically infected with *M. hyorhinis*, and Graffi hamster cells, infected by *M. orale*, served as positive controls. Animal cells were routinely grown in Eagle's minimum essential medium with 10% fetal bovine serum in an atmosphere of 5% carbon dioxide.

Enzyme Assays. To prepare cell extracts for enzyme assays, cells were scraped from the bottles by a rubber policeman and suspended in 10 mM Tris·HCl or phosphate buffer (pH 7.5).

Cells were disrupted by sonication at 4° for a total of 10 sec. The extracts were centrifuged for 40 min at 25,000 × *g*, and the supernatants were assayed for adenosine phosphorylase. The 200 μl reaction mixture, containing 50 μl of 10 mM

TABLE 2. *Formation of organic phosphate in the presence of inorganic ^{32}P and adenosine by mycoplasmas*

Mycoplasma	Control	CH-19	PG20	G145	FH
^{32}P + adenosine	611*	3370	2000	1680	371
− adenosine	693	732	797	418	396

Fifty microliters of 10 mM Tris·HCl buffer (pH 7.4), 50 μl of 0.1 mM adenosine, 50 μl of 0.1 mM $Na_2H^{32}PO_4$ (3.45×10^5 cpm), and 50 μl of mycoplasma were incubated for 20 min at 37°. The reaction was stopped by chilling in an ice bath and adding 10 μl of 60% perchloric acid. Then 50 μl of 80 mM ammonium molybdate and 10 μl of 0.8 M triethylamine were added and the mixture was centrifuged for 10 min at 7000 rpm. Ten microliters of the supernatant solution was counted with 5 ml of Bray's solution. Mycoplasmas were prepared and isolated as described in *Materials and Methods*. Each sample contained: control, 2.58; CH-19, 4.25; PG-20, 3.78; G145, 3.13; and FH, 4.15 mg/ml of protein. Control was broth culture without inoculation of mycoplasma treated by the same procedures. The values are average radioactive counts of two samples.

phosphate buffer (pH 7.4), 50 μl of 1 μM adenosine, 50 μl of enzyme source, and 50 μl of water, was incubated for 20 min at 37°. The reaction was stopped by chilling in ice and addition of 70 μl of 60% perchloric acid. One hundred fifty microliters of 5 M KOH were then added, and the mixture was allowed to stand in ice for 15 min, followed by centrifugation for 10 min at 3000 rpm in an IEC centrifuge. Aliquots of 10 μl were spotted on thin layer films of cellulose powder (MN300), manufactured by Macherey J. Nagel Co., Duren, West Germany, obtainable from Brinkmann Instruments, Westbury, N.Y.

To develop thin-layer chromatograms, the following solvents were used: solvent no. 1, consisting of *n*-butanol/methanol/water/concentrated ammonia (60:20:20:1 v/v), and solvent no. 4, 1.8 M ammonium formate with 2% boric acid, pH 7.0. After 1.5–2.0 hr of development, the films were dried by hot air. Standards were included in each chromatogram. Spots located by ultraviolet quenching were removed from the plates and put into 0.5 ml of H_2O or 0.4 M NH_4OH for 1 hr at room temperature, followed by the addition of 15 ml of Bray's solution (14). Radioactivity was determined in a Beckman LS-350 scintillation system. Assay of organic phosphate formation in the presence of inorganic ^{32}P and adenosine was performed by the method of Sugino and Miyoshi (15). Radioisotopes were purchased from New England Nuclear (Boston, Mass.), and included [2,8-^{3}H]adenosine, [8-^{3}H]adenine, and $Na_2H^{32}PO_4$. Other chemicals were obtained from Sigma (St. Louis, Mo.).

RESULTS

We found that cells infected with *M. hyorhinis* formed adenine from adenosine (Fig. 1). Adenine formation was proportional to protein concentration (Fig. 1A) and time (during 30 min of incubation) (Fig. 1B). Reduction of the substrate, [^{3}H]-adenosine, was also shown to be proportional to protein concentration and incubation time, approaching a stoichiometric relationship with adenine formation. This was not exact because 5–10% of radioactivity was converted to inosine and hypoxanthine, which are the main products of adenosine in uncontaminated cells (data not shown). However, no animal cell tested made adenine without contamination by mycoplasmas and other micro-organisms. This reaction requires phosphate (Table 1). After incubation with dialyzed cell extracts for 20 min at 37°, 65–75% of the substrate adenosine was converted to adenine in the presence of phosphate when cells infected by *M. hyorhinis* were used as an enzyme source. Without phosphate, the reaction was quite slow (2–8%), suggesting phosphorolysis, not hydrolysis, of the nucleoside in this reaction. Human and mouse cells not contaminated by known mycoplasmas or other micro-organisms showed no adenine formation with or without phosphate. However, these cells produced 20–30% inosine and 20–30% hypoxanthine in the presence of phosphate, and 30–40% inosine and a few percent of hypoxanthine without phosphate, suggesting that adenosine deaminase and inosine phosphorylase activity was present in these extracts. Despite the presence of these enzymes in the mycoplasma-contaminated cells, adenosine phosphorylase activity of the mycoplasma dominated the use of the substrate.

TABLE 3. *Reverse reaction: adenosine formation by mycoplasmas from adenine and ribose-1-phosphate*

Mycoplasma	CH-19					FH
Enzyme volume (μl)	5	10	25	25	25	25
Incubation time (min)	20	20	20	10	20	20
[^{3}H]Adenine + ribose 1-phosphate	232*	383	753	445	817	0
[^{3}H]Adenine − ribose 1-phosphate	0	0	0	0	0	0

CH-19 (4.25 mg/ml of protein in 10 mM Tris·HCl, pH 7.4) and FH (4.15 mg/ml of protein in 10 mM Tris·HCl, pH 7.4) were used as enzyme source. The assay conditions are as follows: 50 μl of 10 mM Tris·HCl (pH 7.4), 50 μl of 0.5 mM [^{3}H]adenine, 50 μl of 1 mM ribose-1-phosphate, and 5–25 μl of mycoplasmas (200 μl of final volume in the reaction mixture) were incubated for 10–20 min at 37°. The reaction was stopped by chilling in an ice bath and adding 70 μl of 60% cold perchloric acid. Then 150 μl of 5 M KOH was added, and the mixture was kept for 15 min in an ice bath. The precipitates were removed by centrifugation for 10 min at 3000 rpm in an IEC. 5 μl of the supernatant solution was put on thin-layer plates and developed by no. 4 solvent with carrier adenosine.

* The values show radioactive counts of the adenosine spot (total input on thin-layer plates was 1200 cpm).

To clarify and study the presence of adenosine phosphorylase activity in mycoplasmas without background from eukaryotic cell enzymes, a series of mycoplasmas were cultivated in broth, and extracts prepared as described in *Materials and Methods*. By such crude enzyme preparations, inorganic ^{32}P was converted to organic phosphate in the presence of adenosine (Table 2). The enzyme from CH-19, PG20, and G 145 strains clearly formed organic phosphate in the presence of adenosine, while organism-free broth and the FH strain had no such activity. If the organic phosphate was formed by adenosine phosphorylase activity, the product should be ribose-1-phosphate. We confirmed this by the reverse reaction, that is, adenosine formation from adenine and ribose-1-phosphate using the CH-19 strain enzyme. The results are shown in Table 3. [^{3}H]Adenine and ribose-1-phosphate formed adenosine using CH-19 extracts, but not FH extracts. This reaction was proportional to protein concentration and time of incubation. In the absence of ribose-1-phos-

TABLE 4. *Reaction with adenosine by mycoplasmatales*

Mycoplasmatales		Adenosine converted to
Species	Strain	
Acholeplasma laidlawii	PG 8	Adenine
Spiroplasma citri	R8-A8	
Mycoplasma arginini	G-230	
M. hominis	PG 21	
M. salivarium	PG 20	
M. orale	CH 19	
M. buccale	CH 20	
M. faucium	DC-333	
M. fermentans	PG-18	
M. hyorhinis	BTS-7	
M. gallisepticcum	PG 31	
M. agalactiae var. bovis	70–213	
M. sp.	G 145	
M. lipophilium	MABY Jones	Inosine
M. pneumoniae	FH	None
M. sp.	70–159	

Fifty microliters of 10 mM phosphate (pH 7.4), 50 μl of 10 or 1 μM [^{3}H]adenosine, 50 μl of mycoplasma containing 2–5 mg/ml of protein, and water to make 200 μl of final volume were incubated for 20 min at 37°. The reaction was stopped, and detection and determination of radioactivity were performed as described in the legend of Fig. 1.

phate, no adenosine was formed. The above results collectively indicate that mycoplasmas have adenosine phosphorylase activity catalyzing the following reaction:

$$\text{Adenosine} + \text{phosphate} \rightleftharpoons \text{Adenine} + \text{ribose 1-phosphate}$$

We conclude that the mycoplasmas that commonly contaminate cell cultures contain this enzyme, although *M. pneumoniae*, strain FH, showed lack of this activity. Consequently, we studied this enzyme systematically in a series of mycoplasmatales. Table 4 summarizes the results. In this series, all were positive for adenosine phosphorylase with two exceptions. The first, *M. lipophilium*, produced not adenine but inosine, which is similar to results with eukaryotic cells. *M. pneumoniae* (FH strain) and *M. sp.* strain 70-159 gave no reaction with adenosine under the conditions tested. Although taxonomic and phylogenetic implications of these findings are of interest, one immediate application is the establishment of a simple, rapid, and sensitive assay for detection of mycoplasma contamination in animal cell cultures. We emphasize that most mycoplasmas commonly detected in contaminated cultures have the ability to produce adenine from adenosine. An example is demonstrated in Table 5. Some cultures of hamster tumor cells (12) had been contaminated by *M. orale*. The cells free from demonstrable mycoplasmas and those contaminated by *M. orale* were cultivated separately in plastic petri dishes (2 cm diameter) with 2 ml of Eagle's minimum essential medium with 10% fetal bovine serum. After 2 days of medium exchange, 1 ml of the culture media was removed from the plates and then 0.1 ml of 10 μM [^{3}H]adenosine was added to the plates, which were further incubated for 20 min at 37° in a 5% CO_2 flushing incubator. Two hundred microliters of labeled medium from the plates was transferred into glass tubes on ice and assayed for adenine

TABLE 5. *A simple, rapid, and sensitive method for detection of mycoplasma contamination in cell culture: adenine formation from adenosine in culture medium*

	Products			Substrate
	Adenine	Hypoxanthine	Inosine	Adenosine
Medium from hamster cell culture	0	8	42	55
Medium from hamster cell culture contaminated by *M. orale*	80	11	1	8

Hamster tumor cells (12) free from mycoplasma contamination and cultures contaminated by *M. orale* were separately cultivated to confluency in plastic petri dishes (2 cm diameter) with 2 ml of Eagle's minimum essential medium with 10% fetal bovine serum. One milliliter of the cultured medium was removed from the plates and then 0.1 ml of 10 μM [^{3}H]adenosine was added to the plates, which were further incubated for 20 min at 37° in a 5% CO_2-flushing incubator. Two hundred microliters of medium aliquots from cell-cultured plates were put into glass tubes in ice. We then proceeded as described in the legend of Fig. 1. Values indicate percentage of total radioactivity on thin-layer plates.

formation. As shown in Table 5, by 20 min of incubation, 80% of the adenosine was converted to adenine and 11% to hypoxanthine, while the control culture produced mainly inosine and *no adenine* at all. The same results (adenine and some hypoxanthine) were observed in all mycoplasma contaminated cultures (mostly *M. hyorhinis*, *orale*, and *arginini*), while all control cells (human, monkey, mouse, rat, hamster, cat, rabbit, and viper) failed to produce adenine under these assay conditions.

DISCUSSION

Although recent experiments suggest that several mammalian cells can convert adenine to adenosine at low rates in the presence of ribose-1-phosphate (16), studies of the metabolism of generally labeled adenosine in rat erythrocytes provided no evidence for phosphorolysis of adenosine (16). Adenine has not been detected in normal serum (17) or tissues *in vivo*, except in ischemia (18), although a mammalian adenosine cleaving activity has been suggested from studies of long-established cell cultures (11). Recent studies clearly indicate that deamination is the major route of adenosine metabolism in mammalian cells (19, 20). Despite the fact that Snyder and Henderson (19) showed the formation of adenine from deoxyadenosine, several attempts to form adenine from adenosine were not successful, even in the presence of coformycin (inhibitor of adenosine deaminase), and 2,6-dichloro-9-(tetrahydropyran-2-yl)-9H-purine (inhibitor of adenine phosphoribosyl transferase). The cleavage of adenosine to adenine reported for Ehrlich ascites tumor cells (19) thus may be a result of mycoplasma, which is almost unavoidable in long-term cultures. In our assay conditions, cell cultures without mycoplasma contamination failed to form adenine from adenosine but did convert adenosine to inosine and hypoxanthine, while cells contaminated by mycoplasma formed adenine almost exclusively, with only a slight amount of hypoxanthine. Although the precise mechanisms remain to be established, the possibility of enzyme affinity for adenosine in the presence

of low concentration of the substrate may influence the overall picture.

One application of this study is a detection of mycoplasma contamination in cell cultures. Plates can be screened and easily tested under the conditions shown in Table 5. Since mycoplasmas are located mostly in medium or attached to cell membranes, added substrates are immediately consumed by the mycoplasma enzyme; thus detection of adenine in the medium provides an additional assay for screening of mycoplasma contamination in cell culture.

Concerning the pyrimidine counterparts of nucleic acid metabolism by mycoplasma, the activity of pyrimidine phosphorylase was reported (21) and has been used as a screening method (7). Also, enhanced uracil uptake based on the presence of uracil phosphoribosyl transferase in mycoplasma (M. Hatanaka and C. Long, submitted for publication) may compliment the purine counterparts for the screening of mycoplasma contamination. Schneider *et al.* recently reported the unique incorporation ratio of uracil and uridine in RNA extracted from mycoplasma-contaminated cells (22).

Finally, some tentative phylogenetic and taxonomic speculations emerge from this study. In prokaryotes, adenosine phosphorylase activity was previously observed unequivocally in *E. coli*, *S. typhimurium*, and now in mycoplasmatales, but not in *B. cereus*, suggesting that among Schizomycetes, mycoplasmatales appear closer phylogenetically to Enterobactericeae than to Bacillaceae within the eubacteriales.

The patterns of nucleoside metabolism should contribute to the methods of mycoplasma classification. Depending on their utilization of adenosine, mycoplasmas can be divided into three groups. The first group consists of 13 of the 16 Mycoplasma, Acholeplasma, and Spiroplasma species tested; this group converts adenosine to adenine by phosphorylation. *M. lipophilium* alone falls into the second group with its production of inosine by the deamination pathway characteristic of animal cells. The third group fails to metabolize adenosine and is composed of *M. pneumoniae* and *M. sp.* HRC 70–159. More extensive studies are necessary before one can speculate on the phylogenetic significance of these observations. However, the conversion of nucleosides clearly offers additional characters upon which to classify Mycoplasmatales.

Note Added in Proof. The following strains should read: 70-159 as HRC 70-159; 70-213 as HRC 70-213; CH-19 as CH 19299; CH-20 as CH 20247.

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