Distinctions in *Mollicutes* Purine Metabolism: Pyrophosphate-Dependent Nucleoside Kinase and Dependence on Guanylate Salvage

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Cell preparations of *Acholeplasma florum* L1^T, *Acholeplasma axanthum* S743^T, *Acholeplasma granularum* BTS-39^T, *Spiroplasma floricola* 23-6^T, *Mycoplasma gallisepticum* S6, and *Mycoplasma arginini* G230^T were examined for 13 cytoplasmic enzyme activities involved in the salvage and interconversion of nucleobases, nucleosides, and 5'-mononucleotides. The unique pyrophosphate-dependent nucleoside kinase activity known only in *Acholeplasma laidlawii* B-PG9 was found in *A. axanthum* and *A. granularum*. All the organisms could be divided into three groups based upon their patterns of purine salvage and interconversion activities. All the tested organisms, *A. laidlawii*, and apparently *Mycoplasma mycoides* subsp. *mycoides* lack the ability to synthesize guanylates from other purine mononucleotides, indicating that these members of the class *Mollicutes* can only salvage guanylates. The plant epiphytes *A. florum* and *S. floricola* had an identical purine enzyme pattern which was different from those of all other members of the *Mollicutes* studied.

Mollicutes (mycoplasmas) is a class of procaryotes characterized by lack of a cell wall, complex nutritional requirements, and small genome of generally low guanine-plus-cytosine ratio. Nutritional studies on representatives from each of the three families within the class demonstrate the absolute requirement for nucleobases or nucleosides for growth (3, 5, 8, 11, 17-21, 23). The nutritional requirement for purine precursors suggests the lack of a functional pathway for the de novo synthesis of purines and the dependence on salvage and interconversion enzymes for the biosynthesis of purine nucleotides (11, 24). Hamet et al. (6) examined purine salvage but not nucleotide interconversion activity in a number of *Mycoplasma* species. Only in *Mycoplasma mycoides* subsp. *mycoides* (11, 12), a species not generally available for study in the United States, and *Acholeplasma laidlawii* B-PG9 (24) have the pathways involved in purine salvage and interconversion been completely described.

Here we describe our examination of the class *Mollicutes* for the unique pyrophosphate (PP_i)-dependent nucleoside kinase activity which is only known in *A. laidlawii* B-PG9 (24). When first reported, we speculated that the PP_i-dependent enzyme activity might prove useful in the study of the phylogenetic relationships of the *Mollicutes*. We now have studied six other representative *Mollicutes* for this PP_i-dependent activity and for 12 other cytoplasmic enzyme activities involved in the salvage and interconversion of purine bases, ribonucleosides, and 5'-monophosphates.

MATERIALS AND METHODS

Organisms. *Acholeplasma florum* L1^T, *Acholeplasma axanthum* S743^T, *Acholeplasma granularum* BTS-39^T, and *Spiroplasma floricola* 23-6^T were obtained from J. G. Tully, National Institutes of Health, Frederick, Md. *Mycoplasma gallisepticum* S6 and *Mycoplasma arginini* G230^T were obtained from our stock collection.

Media and growth conditions. All organisms were grown in

our modification of Edward medium (1). Media were supplemented with 2% (vol/vol) (*acholeplasmas*) or 5% (vol/vol) (*mycoplasmas* and *spiroplasma*) heat-inactivated horse serum (lot number 200031; KC Biologicals, Lenexa, Kans.). Media for the growth of *M. arginini* were supplemented with 0.3% (wt/vol) L-arginine hydrochloride (Sigma Chemical Co., St. Louis, Mo.). Organisms were incubated statically at 37°C until mid-log phase of growth (1 to 3 days). Cells were harvested as previously described (1, 14). Cells in dilute (1:20) buffer (13) were lysed osmotically or sonicated for 1- to 30-s pulses (Sonifier Cell Disruptor; Heat Systems, Melville, N.Y.) while on wet ice. Cell extracts were fractionated by centrifugation, dialyzed, and prepared as we described for *A. laidlawii* B-PG9 (14, 24). Purity of the fractions was monitored by localization of reduced nicotinamide adenine dinucleotide oxidase (13, 14) and adenosine triphosphatase (EC 3.6.1.3) (24) activities.

Assay. The activities of 13 cytoplasmic enzymes involved in the salvage and interconversion of nucleobases, nucleosides, and 5'-mononucleotides were assayed as described for *A. laidlawii* B-PG9 (24). Briefly, ¹⁴C-labeled purine substrates were mixed with cofactors at pH 7.5. Dialyzed cell extract containing 20 to 40 µg of protein (2) was added to start the reaction. The reaction mixtures were then incubated at 37°C with shaking for 4 to 60 min. Incubation times were selected such that greater than 50% of the starting labeled substrate remained at the end of the reaction period. In preliminary trials, the velocity of each of the reactions was shown to be linear over the prescribed time period and conditions (24). Reactions were terminated by heating at 95°C for 2 min. Samples (20 µl) of the heated reaction mixtures were spotted onto polyethyleneimine-cellulose plates (Analtech, Inc., Newark, Del.) with 10 µg each of nonradioactive standards (24). ¹⁴C-labeled substrate and product were separated by thin-layer chromatography in polar solvents (17, 24). Resolved purines were visualized by ultraviolet light, scraped into counting fluid, and assayed for radioactivity. In all cases, greater than 90% of the applied label was recovered from substrate and product spots. During initial experiments, all plates were autoradiographed after chromatography to help exclude the presence of competing reactions. Radioisotope data were corrected for

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TABLE 1. Enzyme activity

Enzyme and substrate	Enzyme activity ^a in:						
	<i>A. laidlawii</i> B	<i>A. axanthum</i>	<i>A. granularum</i>	<i>A. florum</i>	<i>S. floricola</i>	<i>M. gallisepticum</i>	<i>M. arginini</i>
Phosphoribosyltransferases							
ADE	5.08 (1.45)	6.09 (1.31)	8.34 (1.95)	47.0 (3.84)	16.1 (1.79)	1.18 (0.309)	10.7 (0.324)
GUA	2.76 (1.66)	9.57 (0.770)	4.66 (0.512)	2.64 (0.614)	16.3 (0.923)	18.3 (2.36)	2.11 (0.096)
HX	3.69 (2.11)	6.66 (0.998)	2.49 (0.458)	2.55 (0.331)	6.03 (1.95)	7.16 (1.06)	2.97 (0.014)
X	0.08 (0.02)	NA ^b	NA	NA	NA	NA	NA
Nucleoside phosphorylases							
ADO	1.97 (0.62)	2.97 (0.070)	0.445 (0.028)	1.90 (0.271)	3.41 (0.430)	1.01 (0.171)	2.48 (0.186)
GUO	1.30 (0.27)	1.27 (0.045)	0.135 (0.009)	4.93 (0.289)	3.15 (0.455)	0.663 (0.101)	5.88 (1.06)
INO	1.30 (0.49)	1.11 (0.070)	0.639 (0.115)	2.19 (0.462)	3.93 (0.750)	0.565 (0.257)	1.65 (0.121)
XO ^c	0.009 (0.003)	NA	NA	NA	NA	NA	NA
Nucleoside phosphorylases							
ADE	7.12 (3.54)	11.5 (0.044)	11.1 (1.36)	93.6 (8.68)	26.5 (4.58)	18.7 (2.69)	15.6 (1.03)
GUA	13.2 (4.13)	10.1 (1.73)	17.0 (1.30)	17.7 (2.70)	37.8 (7.58)	14.9 (4.43)	9.74 (1.20)
HX	7.56 (1.89)	6.35 (0.473)	6.07 (0.0119)	4.24 (1.05)	14.3 (0.318)	3.49 (0.802)	3.39 (0.215)
X	0.034 (0.007)	NA	0.012 (0.002)	NA	0.645 (0.057)	NA	NA
PP _i -nucleoside kinases							
ADO	58.9 (21.3)	0.529 (0.160)	0.161 (0.012)	NA	NA	NA	NA
GUO	50.8 (21.3)	0.291 (0.080)	NA	NA	NA	NA	NA
INO	32.3 (8.68)	NA	NA	NA	NA	NA	NA
XO	NA	NA	NA	NA	NA	NA	NA
5'-Nucleotidases							
AMP	12.8 (2.76)	0.810 (0.163)	0.039 (0.002)	NA	NA	0.064 (0.009)	NA
GMP	0.15 (0.020)	0.068 (0.001)	0.010 (0.001)	NA	NA	NA	NA
IMP	1.82 (0.120)	0.064 (0.015)	0.022 (0.002)	NA	NA	NA	NA
XMP	0.003 (0.001)	NA	NA	NA	NA	NA	NA
Adenylosuccinate synthetase and lyase	0.524 (0.184)	0.210 (0.010)	0.035 (0.005)	NA	NA	0.108 (0.027)	0.145 (0.01)
GMP reductase	0.642 (0.301)	0.050 (0.003)	0.008 (0.001)	0.003 (0.001)	0.037 (0.005)	NA	NA
GMP synthetase	NA	NA	NA	NT ^d	0.005 (0.001)	NA	NA
IMP dehydrogenase	NA	NA	NA	NA	NA	NA	NA
Adenylate deaminases (AMP, ADO, ADE)	NA	NA	NA	NA	NA	NA	NA

^a Values are given in nanomoles of product synthesized per minute per milligram of protein ($\pm$ standard deviation). The number of different cell batches studied is three except for *A. laidlawii* B. The data listed for *A. laidlawii* B are our previous results (24).

^b NA, No activity detected (<0.003 nmol of product synthesized $\text{min}^{-1} \text{mg}^{-1}$ of protein) in extracts from each of three or more different batches of cells.

^c XO, Xanthosine.

^d NT, Not tested.

quenching and calculated as disintegrations per minute and then converted to nanomoles of product synthesized per minute per milligram of protein by calculation with the specific activity of the labeled substrate.

RESULTS

The various members of the *Mollicutes* were tested for activities associated with the salvage and interconversion of purine bases, ribonucleosides, and ribomononucleotides. Three to seven different batches of each organism were assayed. Enzyme activities are reported as nanomoles of product per minute per milligram of protein (Table 1). Enzyme activities were considered positive when the rate of product synthesized exceeded $0.003 \text{ nmol min}^{-1} \text{mg}^{-1}$ of protein. The relationship of the various enzymes involved in purine salvage and interconversion is shown in Fig. 1A and B. The novel PP_i-dependent purine nucleoside kinase activity found in *A. laidlawii* B (24) was detected in *A. axanthum* for adenosine (ADO) and guanosine (GUO) and in *A. granularum* for ADO. No PP_i-dependent xanthosine nucleoside kinase activity or adenosine 5'-triphosphate (ATP)-dependent ADO, inosine (INO), or GUO kinase activity (adenosine kinase [EC 2.7.1.20] and inosine kinase [EC 2.7.1.73]) was detected in any organism. Phosphoribosyltransferase activity (adenine [EC 2.4.2.7] and hypoxanthine [EC 2.4.2.8]) for the substrates adenine (ADE),

guanine (GUA), and hypoxanthine (HX) were found in all the organisms. Phosphoribosyltransferase activity for xanthine (X) was not detected in any organism in this study but was detected previously in *A. laidlawii* B (24). Nucleoside phosphorylase activity (purine-nucleoside phosphorylase [EC 2.4.2.1]) in the direction of the nucleobase with ADO, INO, and GUO or in the opposite direction toward the nucleoside with ADE, HX, or GUA was found in all organisms. In *A. granularum* we also detected nucleoside phosphorylase activity for X. Cytoplasmic 5'-nucleotidase (EC 3.1.3.5) activity for adenosine 5'-monophosphate (AMP), guanosine 5'-monophosphate (GMP), and inosine 5'-monophosphate (IMP) was detected in *A. axanthum* and *A. granularum*. No cytoplasmic 5'-nucleotidase activity was detected in *S. floricola* or *A. florum* or either *Mycoplasma* species. Adenylosuccinate synthetase (EC 6.3.4.4) and adenylosuccinate lyase (EC 4.3.2.2) were detected in all organisms except *S. floricola* and *A. florum*. GMP reductase activity (EC 1.6.6.8) was found in the achleoplasmas and the spiroplasma but not in the two *Mycoplasma* species. GMP synthetase activity (EC 6.3.4.1) was detected only in *S. floricola*. IMP dehydrogenase (EC 1.2.1.14), AMP deaminase (EC 3.5.4.6), adenosine deaminase (EC 3.5.4.4), and adenine deaminase (EC 3.5.4.2) activities were not detected in any organism. Enzyme activity for these latter four enzymes was detected in *Escherichia coli* ATCC 25922,

FIG. 1. Schematic diagram of the proposed purine salvage pathways in six members of *Mollicutes*. The data are all taken from Table 1. Abbreviations: 1, adenine phosphoribosyltransferase; 2, hypoxanthine-guanine phosphoribosyltransferase; 3, purine nucleoside phosphorylase; 4, 5'-nucleotidase; 5, PP_i-dependent nucleoside kinase; 6, adenylosuccinate synthetase; 7, adenylosuccinate lyase; 8, GMP reductase; PRPP, phosphoribosyl pyrophosphate; R-I-P, ribose-1-phosphate; and AS, adenylosuccinate. (A) *A. granularum* and *A. axanthum*. In panel A, PP_i-guanosine nucleoside kinase activity (PP_i-dependent nucleoside kinase) was found only in *A. axanthum*. Not included in panel A is the observation that *A. granularum* had xanthine phosphorylase activity and *S. floricola* had GMP synthetase activity. (B) *A. florum*, *S. floricola*, *M. arginini*, and *M. gallisepticum*. Panel B shows that only *M. arginini* and *M. gallisepticum* have enzyme activities for adenylosuccinate synthetase and adenylosuccinate lyase, while only *A. florum* and *S. floricola* have enzyme activity for GMP reductase.

Bacillus subtilis (60015) (24), or human erythrocytes and lymphocytes (data not shown) (24).

DISCUSSION

We found the novel PP_i-dependent nucleoside kinase activity described in *A. laidlawii* B-PG9 (24) in *A. axanthum* and *A. granularum* but not in *A. florum*. Similarly, we detected the cytoplasmic 5'-nucleotidase activity reported in *A. laidlawii* B-PG9 (24) in *A. axanthum* and *A. granularum* but not in *A. florum*. The lack of similarity between *A. florum* and the other achleplasmas was not surprising since (i) *A. florum* shows little homology with other achleplasmas or mycoplasmas when examined by nucleic acid hybridization (22); (ii) it is also relatively unrelated to other *Achleplasma* species, as judged by growth inhibition and plate immunofluorescence studies (22); (iii) unlike other achleplasmas, it synthesizes little lipid from acetate (15); and (iv) unlike other achleplasmas, it has significant levels of reduced nicotinamide adenine dinucleotide oxidase activity localized in the cytoplasm (J. D. Pollack, K. D. Beaman, and V. V. Tryon, Abstr. Meet. Int. Organ. Mycoplasma. 1982, Yale J. Biol. Med. 57:891). The findings of PP_i-dependent nucleoside kinase and 5'-nucleotidase activity in the cytoplasm of three of four achleplasmas, including *A. laidlawii* B-PG9 (24) but not *A. florum*, suggests that these activities may be common to other *Achleplasma* species and that further study of *A. florum* and other plant isolates

and their taxonomic relationship to other achleplasmas is warranted.

Most striking in the examination of the purine salvage and interconversion enzyme activities of *Achleplasma*, *Mycoplasma*, and *Spiroplasma* species is the lack of enzyme activities necessary for the synthesis of guanylates from other purine mononucleotides. In the organisms tested here, and as has been reported for *M. mycoides* subsp. *mycoides* (11, 12), no activity was found for AMP deaminase or IMP dehydrogenase. AMP deaminase permits the conversion of AMP to IMP, and IMP dehydrogenase permits the conversion of IMP to xanthosine 5'-monophosphate (XMP). XMP is converted by GMP synthetase to GMP. Only *S. floricola* exhibited GMP synthetase activity, although as indicated above, like every other member of *Mollicutes* studied, it lacked IMP dehydrogenase activity. Therefore, some members of *Mollicutes* studied can convert guanylates to inosinates, but none of the six studied can apparently convert adenylylates to inosinates or inosinates to guanylates. These six members of *Mollicutes* and *A. laidlawii* B-PG9 may only be able to salvage guanylates.

The organisms we studied are divided into three groups based on their respective patterns of purine salvage and interconversion enzyme activities in cytoplasmic fractions. All the achleplasmas tested except for *A. florum* had some PP_i-dependent nucleoside kinase activity and cytoplasmic 5'-nucleotidase activity. The features of this pattern are shown in Fig. 1A and are very similar to those already

published for *A. laidlawii* B-PG9 (24). The main difference is the ability of *A. laidlawii* B-PG9 to interconvert xanthylates (24). A second pattern of purine salvage was displayed by *A. florum* and *S. floricola* (Fig. 1B). *A. florum*, which was isolated from the surfaces of plants (9), had a purine enzyme pattern identical to that of *S. floricola*, another plant epiphyte but included in the family *Spiroplasmatales* (4). Like the achleoplasmas studied, both *A. florum* and *S. floricola* could convert GMP to IMP. Like the two *Mycoplasma* species, each lacked purine nucleoside kinase and 5'-nucleotidase activity. The absence of these two latter activities indicates that the flow of carbon is towards GMP via the guanine phosphoribosyltransferase reaction which requires phosphoribosylpyrophosphate. This hypothesis emphasizes the role of phosphoribosylpyrophosphate in *Mollicutes* purine metabolism (16).

M. arginini and *M. gallisepticum* exhibited a third pattern of purine salvage which was very similar to that for *A. florum* and *S. floricola* (Fig. 1B). The two *Mycoplasma* species had adenylosuccinate synthetase and lyase activities but no GMP reductase activity. The converse was true for *A. florum* and *S. floricola*.

Our inability to detect enzyme activity does not necessarily mean that these enzymes are inactive or absent in the intact organism. During lysis and subsequent fractionation, enzyme activity may be lost or our assays may lack some necessary cofactor or condition. However, our data are consistent with the whole cell-labeling experiments of McIvor and Kenny (10) and Mitchell and Finch (11) and with most of the known nutritional requirements of these organisms.

Enzyme activities are reported here as rates; however, such values may be in error, since our assays were conducted with crude cell extracts, and there may be competing reactions. We minimized competing reactions by extensive dialysis of our cell-free preparations and adjustment of the reaction times (24). In the case of IMP dehydrogenase activity, we recovered at least 97% of the radioactivity as unreacted IMP; that is, there were no competing reactions. Therefore, our failure to detect IMP dehydrogenase activity was not due to the conversion of XMP, the product of the reaction, to other compounds or back to IMP since there is no known back reaction. When testing was possible, control reactions lacking required cofactors also showed complete recovery of substrate. We used *E. coli* as our positive control for IMP dehydrogenase activity (24).

No ATP-dependent purine nucleoside kinase activity was detected in any member of *Mollicutes*. To determine whether the lack of ATP-dependent activity was due to contaminating membrane adenosine triphosphatase activity, dialyzed cell extract was examined for adenosine triphosphatase activity. Under conditions described for the assay of *A. laidlawii* B-PG9 (24) purine nucleoside kinase activity, greater than 90% of the radiolabeled ATP remained after the incubation period (data not shown). This indicates that our inability to detect ATP-dependent purine nucleoside kinase activity was not due to the loss of ATP in the reaction mixtures as a result of contaminating membrane adenosine triphosphatase activity.

This is the first report suggesting the dependence of members of the *Mollicutes* on guanine phosphoribosyltransferase activity. Since IMP dehydrogenase activity is apparently absent in all members of the *Mollicutes* tested, we conclude that the class *Mollicutes* cannot synthesize guanine monophosphate from other purine mononucleotides. These organisms are, therefore, dependent on

PP_i-dependent guanosine kinase and guanine phosphoribosyltransferase (in some species only the latter) for the biosynthesis of GMP. Although we cannot yet draw too broad a generalization, the data from the seven members of the *Mollicutes* we examined suggest that guanine phosphoribosyltransferase and possibly PP_i-dependent guanosine kinase are essential *Mollicutes* enzymes and promising loci for biochemical inhibition which may alter infection, pathogenesis, or pathology caused by members of the *Mollicutes*, for example, in the avian pathogen *M. gallisepticum*. The swine pathogen *Mycoplasma hyopneumoniae* J, although not studied extensively, lacks IMP dehydrogenase activity (≤ 3 pmol of XMP synthesized min⁻¹ mg⁻¹) in each of three different cell batches (J. D. Pollack, unpublished data) and may be dependent on guanylate salvage and guanine phosphoribosyltransferase activity. Similarly, *M. mycoides* subsp. *mycoides* also lacks IMP dehydrogenase activity and may be dependent on guanylate salvage and guanine phosphoribosyltransferase activity (11). Inhibitors of guanine phosphoribosyltransferase activity have been described, but nonspecific effects have made them unsuitable for use in vivo in humans (7). Perhaps if our observations are more widespread amongst the members of the *Mollicutes*, these inhibitors may have the potential for use in the treatment of *Mollicutes* infections in animals, cell cultures, or perhaps plants.

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