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Pathways of Pyrimidine Deoxyribonucleotide Biosynthesis in *Mycoplasma mycoides* subsp. *mycoides*

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By measuring the specific activity of deoxyribonucleotides isolated from DNA after the incorporation of ^{14}C-labeled precursors with and without competition from other nucleotide precursors, we defined the major pathways of pyrimidine deoxyribonucleotide synthesis in *Mycoplasma mycoides* subsp. *mycoides*. Uracil, guanine, and thymine are required for the synthesis of nucleotides. Cytidine competed effectively with uracil to provide all of the deoxycytidine nucleotide, as well as most of the deoxyribose-1-phosphate, for the synthesis of thymidylate from thymine via thymidine phosphorylase. Each of dUMP, dCMP, and dTMP competed with cytidine for incorporation into DNA thymidylate. Appreciable incorporation of exogenous deoxyribonucleoside 5'-monophosphates into DNA without prior dephosphorylation was observed. Dephosphorylation also occurred since the added deoxyribonucleotide provided phosphate for the synthesis of the other nucleotides in DNA in competition with the $^{32}P_i$ in the growth medium. Hydroxyurea inhibited cell growth and decreased the intracellular level of dATP, consistent with the action of a ribonucleoside diphosphate reductase with regulatory properties similar to those of the *Escherichia coli* enzyme.

The mycoplasmas are the smallest known organisms capable of autonomous growth outside host cells and are characterized by their lack of a cell wall. In keeping with their small size, the genome of mycoplasmas is about 1/5 the size of that of *Escherichia coli* (14). Probably as a result of this economy of genome, they seem to lack many biosynthetic functions since they exhibit complex nutritional requirements for growth in vitro (17). Among such requirements are guanine and uracil for nucleotide synthesis.

We have described pathways for ribonucleotide synthesis in *Mycoplasma mycoides* subsp. *mycoides* (5–7) in which guanine can provide adenine nucleotide via the deamination of GMP and the amination of IMP and uracil can provide cytosine nucleotides via the amination of UTP. Thymine is also a growth requirement for this organism. In most other organisms, thymidine nucleotides arise via the methylation of dUMP by N^5,N^{10}-methylenetetrahydrofolate under the action of thymidylate synthetase (3, 11). Alternatively, salvage synthesis can occur via the phosphorylation of thymidine, which can be derived by the transfer of the deoxyribosyl moiety from deoxyribose-1-phosphate to preformed thymine. The requirement of *M. mycoides* for thymine, together with its limited capacity for folate metabolism (8), suggests the latter pathway for thymine nucleotide synthesis, thus raising the question of the source of deoxyribose-1-phosphate for the synthesis.

The experiments described in this paper were performed to define the pathways of pyrimidine deoxyribonucleotide synthesis in *M. mycoides* and to establish the precursor of thymidylate deoxyribose. To determine the origin of the constituents of *M. mycoides* DNA, we labeled cells with $^{32}P_i$ and ^3H- or ^{14}C-labeled precursors of the base or ribose moieties or both. By measuring the ratio of ^{14}C to ^{32}P incorporated into deoxynucleoside monophosphates (dNMPs) isolated from DNA, we could calculate the specific activity of the ^{14}C radioactivity in these deoxynucleotides. Where this specific activity was lower than that of the labeled precursor, it indicated that the incorporation of the labeled precursor had been diluted by competition from a nonradioactive precursor in the synthesis of the nucleotide.

MATERIALS AND METHODS

Organism and culture medium. Cultures of *M. mycoides* subsp. *mycoides* were grown in medium C2 of Rodwell (18), modified as described previously (5). In all experiments, the medium contained adenine, guanine, uracil, and thymine.

Chemicals and radiochemicals. Cytidine and thymine were from Merck Sharp & Dohme, West Point, Pa., deoxyguanosine and deoxyadenosine were from Boehringer Mannheim Corp., New York, N.Y., and all other unlabeled bases, nucleosides, and nucleotides

were obtained from Sigma Chemical Co., St. Louis, Mo. Tris, phosphodiesterase I from *Crotalus atrox* venom, DNase I from bovine pancreas, and the scintillation solute 2,5-diphenyloxazole were also from Sigma. Bovine serum albumin was from Calbiochem, La Jolla, Calif. Amberlite IRC-50 resin was supplied by Serva. Carrier-free $^{32}P_i$ dissolved in dilute HCl was obtained from the Australian Atomic Energy Commission. [2-^{14}C]cytidine (43 Ci/mol) was from Service des Molécules, Marquées, France. [2-^{14}C]uracil (58 Ci/mol), [*U*-^{14}C]cytidine (485 Ci/mol), and [5-^{3}H]cytidine (31 Ci/mmol) were supplied by the Radiochemical Centre, Amersham, England.

Isolation of deoxyribonucleotides from DNA. Cells from 1.0 ml of culture at the late logarithmic phase were harvested by centrifugation at 8,000 rpm for 10 min, suspended in 2 ml of 1 M NaCl containing 0.5 mg of carrier DNA, and treated with 1 ml of 0.6 M $HClO_4$ for 10 min at 0°C. The acid-insoluble residue was washed once with 2 ml of 0.2 M $HClO_4$ at 0°C, and the washed pellet was incubated in 0.3 ml of 0.3 M KOH for 1 h at 37°C. The mixture was chilled to 0°C, and DNA and protein were precipitated from the alkaline hydrolysate by acidification with cold $HClO_4$ to approximately 0.4 M (4). The precipitate was washed twice with 1 ml of 0.4 M $HClO_4$, redissolved in 1 ml of 2 M potassium acetate, and adjusted to pH 7 at 0°C with 1 M KOH. Protein was precipitated by heating for 15 min at 100°C and removed by centrifugation. DNA was precipitated at −15°C overnight by the addition of 2 volumes of cold ethanol. The precipitated DNA was washed once with 1 ml of 67% ethanol containing 0.1 M $MgCl_2$ and was then dissolved in 135 μl of 50 mM Tris buffer (pH 8.1; 37°C), to which was then added 5 μl of 1 M $MgCl_2$, 10 μl of bovine serum albumin (10 mg/ml in Tris buffer), and 60 Kunitz units of DNase (25 μg of protein) in 25 μl of Tris buffer. The DNA was incubated for 2 h at 37°C before the addition of 0.075 U of phosphodiesterase I (125 μg of protein) in 25 μl of Tris buffer, after which the digestion was continued for a further 30 min. At the completion of digestion, protein was precipitated by heating for 5 min at 100°C and removed by centrifugation. The product dNMPs were separated on polyethyleneimine-cellulose (PEI-C) thin-layer sheets (12, 13) by either one- or two-dimensional chromatography. Deproteinized incubation mixtures were adjusted to 25 mM CH_3COOH, and then 50-μl samples were spotted onto PEI-C chromatograms (10 by 10 cm), dried, and desalted by immersion in anhydrous methanol containing 1.2 g of Tris per liter. The dried chromatograms were developed with 1 M HCOOH in the first dimension, dried, desalted as above, and then developed in the second dimension with 1.7 M ammonium acetate containing 2.4% H_3BO_3, pH 7.0. For the one-dimensional separation, the deproteinized samples were adjusted to 25 mM CH_3COOH–5 mM sodium tungstate, and 25-μl samples were streaked across the origin of PEI-C chromatograms (10 by 2.5 cm), dried, and desalted as above. The dried chromatograms were then developed with 1 M CH_3COOH–25 mM LiCl. Sodium tungstate caused the retention of P_i at the origin of the chromatogram. The regions containing nucleotides were located by their absorption of UV light and by autoradiography of the layer. For elution, the region of the chromatograms containing each of the nucleotides was excised, and the PEI-C was scraped from its plastic backing into a plastic pipette tip plugged with a small amount of nylon wool. This tip was then inserted into a similarly plugged tip, capped at the bottom to prevent solvent flow and containing 100 μl of IRC-50 in distilled water. The nucleotide was then eluted from the PEI-C onto the IRC-50 resin with 300 μl of 1 M magnesium acetate followed by 300 μl of distilled water. The IRC-50 columns were uncapped, and the eluates were collected directly into scintillation vials. A further washing with 400 μl of distilled water was pooled with the eluates. Samples were acidified with 50 μl of 11 M HCl and prepared for counting by the addition of 10 ml of scintillator (5 g of 2,5-diphenyloxazole per liter of toluene; Teric-10, 2:1 [vol/vol]).

Counting techniques. Prepared samples were counted in an LKB Rack Beta model 1215 liquid scintillation spectrometer with settings optimal for discrimination between ^{14}C and ^{32}P or between ^{14}C and ^{3}H.

Estimation of deoxyribonucleoside triphosphates. The growth and labeling of cultures and the extraction of nucleotides were performed as described for ribonucleoside triphosphates (1, 6) with modifications for the estimation of dNTPs (15).

RESULTS

Base composition of DNA from *M. mycoides*. In initial experiments to test the procedures for the isolation and analysis of labeled DNA, we determined the composition of extracted DNA by measuring the ^{32}P radioactivity in the dNMPs derived from the DNA of cells uniformly labeled with $^{32}P_i$. The technique reproducibly gave the nucleotide composition of the DNA as approximately 11.9% dGMP, 12.3% dCMP, 38.4% dAMP, and 37.4% dTMP. A variable amount of $^{32}P_i$, ranging from 0.7 to 2.3% of the total deoxyribonucleotide counts, was also observed. This appeared to arise from a slight breakdown of the dNMPs by contaminant phosphatase activity in the commercial phosphodiesterase I. The nucleotide most affected by this degradation was dTMP.

Utilization of bases and nucleosides. Preliminary studies indicated that of the nucleotides in DNA, only dCMP was labeled by [2-^{14}C]uracil and [2-^{14}C]cytidine. In double-labeling experiments with $^{32}P_i$, [2-^{14}C]uracil contributed all of the cytosine in dCMP, with this contribution decreasing to one-third in the presence of deoxycytidine and to zero with cytidine present. That cytidine in the presence of uracil provides all of the cytosine in DNA is consistent with the previous finding that cytidine under similar growth conditions provides all of the cytidine nucleotide in RNA (5).

To gain information on the origin of the deoxyribose in DNA, double-labeling of cells with $^{32}P_i$ and [*U*-^{14}C]cytidine was employed. Cytidine is used to form cytidine nucleotides (5), and if these are reduced to deoxyribonucleotides as occurs in other organisms, then the labeled ribose moiety of the [*U*-^{14}C]cytidine will

TABLE 1. Effect of added deoxynucleosides on the incorporation of [*U*-^{14}C]cytidine in nucleotides in DNA[a]

Addition to growth medium[b]	Molar ratio of ^{14}C- to ^{32}P-nucleotide from DNA			
	dCMP	dTMP[c]	dGMP[c]	dAMP[c]
None	1.06	0.95	0.14	0.16
None	1.15	0.99	0.15	0.17
Deoxycytidine	0.81	0.77	0.12	0.17
Deoxyuridine	1.06	0.74	0.15	0.17
Deoxythymidine	1.10	0.90	0.15	0.15
Deoxyadenosine	1.00	0.80	0.06	0.02
Deoxyguanosine	0.99	0.81	0.00	0.07

[a] From a small inoculum, cultures were grown with [*U*-^{14}C]cytidine, $^{32}P_i$, and unlabeled deoxynucleosides as indicated. At the late logarithmic phase, they were sampled for isolation and counting of deoxyribonucleotides from DNA, as described in the text.

[b] The concentration of deoxynucleosides was 80 μM.

[c] The molar ratio of ^{14}C- to ^{32}P-nucleotides, assuming the incorporation of ^{14}C label from the five pentose carbons only.

appear in the dCMP isolated from DNA. If the deoxycytidine nucleotides can serve as a source of deoxyribose-1-phosphate for combination with thymine to form thymidine, then label from the cytidine ribose could appear in the dTMP of the DNA.

The results in Table 1 show that label from [*U*-^{14}C]cytidine entered all four nucleotides of the DNA. Since the nucleotides other than dCMP do not incorporate label from [2-^{14}C]cytidine, this labeling must be derived solely from cytidine ribose, appearing as deoxyribose. Cytidine provides all of the nucleoside portion of dCMP, all of the deoxyribose in dTMP, and a much smaller proportion of the deoxyribose in dGMP and dAMP. Confirmation that the pentose from cytidine was incorporated into other nucleotides came from double-labeling experiments with [5-^{3}H]cytidine and [*U*-^{14}C]cytidine. The results indicated that the fractions of the pentose carbon which were derived from cytidine for the nucleotides dCMP, CMP, dTMP, UMP, dAMP, dGMP, AMP, and GMP were, respectively, 1.0, 1.0, 0.90, 0.34, 0.17, 0.17, 0.10, and 0.10.

Competition studies (Table 1) show that deoxyuridine was most effective in excluding the cytidine label from dTMP, whereas deoxycytidine excluded a small amount of cytidine label from dCMP and a considerable amount from dTMP. The other deoxynucleosides excluded less cytidine label from dTMP, but deoxyguanosine and deoxyadenosine almost prevented the occurrence of cytidine label in dGMP and dAMP. Growth studies showed that the thymine requirement of *M. mycoides* can be fully met by thymidine. Labeling studies of cells grown on thymine or thymidine suggested that there were adaptive factors influencing the utilization of the two precursors. It should be noted that the experiments reported in Table 1 used media containing thymine, so that the cells may have been poorly adapted to the use of thymidine.

Utilization of nucleotides. The data in Table 2 show the competitive effect of the dNMP and ribonucleoside NMPs (rNMPs) on the incorporation of $^{32}P_i$ and [*U*-^{14}C]cytidine into nucleotides in DNA. The availability of an exogenous unlabeled dNMP caused a decrease in the incorporation of $^{32}P_i$ into that nucleotide in DNA as compared with its complementary nucleotide and did not affect the ratio in the other pair of

TABLE 2. Effect of added nucleotides on the incorporation of [*U*-^{14}C]cytidine in DNA[a]

Addition to growth medium[b]	[^{32}P]dCMP / [^{32}P]dGMP[c]	[^{32}P]dTMP[c] / [^{32}P]dAMP[c]	Molar ratio of ^{14}C- to ^{32}P-nucleotide from DNA			
			dCMP	dTMP[c,d]	dGMP[d]	dAMP[d]
None	1.00	1.00	1.04	0.88	0.16	0.21
dCMP	0.78	1.06	1.32 (1.00)[e]	0.54 (0.41)	0.22 (0.17)	0.22 (0.17)
dUMP	1.14	0.88	1.37 (1.00)	0.46 (0.34)	0.18 (0.13)	0.19 (0.14)
dTMP	1.04	0.46	1.45 (1.00)	0.77 (0.53)	0.18 (0.12)	0.12 (0.12)
dAMP	0.98	1.46	1.11 (1.00)	0.78 (0.70)	0.11 (0.10)	0.17 (0.15)
dGMP	1.53	1.03	1.45 (1.00)	1.05 (0.72)	0.10 (0.07)	0.25 (0.17)
CMP	0.83	1.10	0.89	0.74	0.19	0.23
UMP	0.87	1.07	1.00	0.73	0.13	0.16
AMP	1.02	1.42	0.95	0.87	0.24	0.26
GMP	1.28	1.07	1.03	0.80	0.15	0.22

[a] See Table 1, footnote *a*.

[b] The concentration of nucleotides was 80 μM.

[c] After correction for 5% depurination during the isolation of DNA (the deoxyribose-5-phosphate resulting from depurination chromatographs with dTMP).

[d] See Table 1, footnote *c*.

[e] The figures in parentheses are corrected for the utilization of unlabeled phosphate from added dNMP (see the text).

nucleotides. This observation is consistent with an uptake and incorporation of the exogenous dNMP into DNA without prior dephosphorylation. The values indicate that of the dCMP, dTMP, dGMP, and dAMP in DNA, 22, 54, 35, and 32%, respectively, were derived from the corresponding exogenous dNMP at 80 μM.

With exogenous dNMPs available, the values for the ratios of ^{14}C-nucleotide to ^{32}P-nucleotide in dCMP from DNA were much greater than in the controls. This increase must arise from the utilization of phosphate from exogenous nucleotide to provide a source of P_i diluting the specific activity of the intracellular $^{32}P_i$. The mechanism for the provision of this diluent P_i cannot be simply hydrolysis and extracellular release of the nucleotide-bound phosphate, since this would only allow a 16% dilution of the specific activity of the $^{32}P_i$. It seems more probable that the nucleotide phosphate is released intracellularly, where it lowers the specific activity of the intracellular $^{32}P_i$ pool to cause the ratios of ^{14}C-nucleotide to ^{32}P-nucleotide to represent overestimates of the contribution of [^{14}C]cytidine to the nucleotide synthesized. Since cytidine contributes all of the nucleoside moiety of dCMP, we might reasonably assume that the ratio of ^{14}C-nucleotide to ^{32}P-nucleotide in dCMP should be equal to 1 and that the extent to which this ratio is greater than 1 is a measure of the dilution of the intracellular $^{32}P_i$ pool by phosphate derived from exogenous dNMP. Values corrected on the basis of this assumption are shown in parentheses in Table 2. In the particular case of added dCMP, this value may be an undercorrection since it does not allow for the likelihood that unlabeled deoxycytidine formed from the dephosphorylation of dCMP would compete with [^{14}C]cytidine as a source of dCMP in DNA, so lowering the observed $^{14}C/^{32}P$ ratio (cf. Table 1).

In view of the corrected values in Table 2, dUMP was the most effective dNMP in competing with cytidine for the supply of deoxyribose for dTMP synthesis, followed by dCMP and dTMP, with dAMP and dGMP considerably less effective. However, a comparison of the effects of dUMP and dCMP would be subject to undercorrection for the value with added dCMP, as noted above. Thus, the effect of dCMP may more closely approximate that of dUMP. It should be noted that dCMP was more effective in contributing to the deoxyribose moiety of DNA thymidylate than in providing DNA deoxycytidylate (as shown by the [^{32}P]dCMP/[^{32}P]dGMP ratio). This was similar to the pattern of the utilization of deoxycytidine. When account is taken of the capacity of dTMP to supply the thymidylate in DNA without prior dephosphorylation (54%), it was, overall, the most effective nucleotide in excluding cytidine pentose from DNA thymidylate.

The addition of rNMP to the growth medium caused some decrease in the incorporation of $^{32}P_i$ into its corresponding nucleotide in DNA as compared with the complementary nucleotide. The values suggest that of the dCMP, dAMP, and dGMP in DNA, 17, 22, and 30%, respectively, were derived from the corresponding rNMP without prior dephosphorylation. Other experiments (data not shown) indicated that there was no such use of exogenous rNMPs for RNA synthesis. The rNMP added to the growth medium had some effect, but less than that of dNMPs, on the incorporation of [^{14}C]cytidine label into dTMP. The indicated utilization of rNMPs to contribute to dNMPs in DNA could arise via ribonucleotide reduction.

Ribonucleotide reduction. To obtain evidence as to whether the reduction of ribonucleotides occurs at the diphosphate or triphosphate level in *M. mycoides*, the effect of hydroxyurea on cell growth was tested (2). Little inhibition of growth occurred with concentrations of hydroxyurea up to 0.1 mM, whereas 1 mM hydroxyurea decreased the growth rate by approximately 41%, and 10 mM hydroxyurea decreased the growth rate by approximately 60%.

To test the possibility that this inhibitory action of hydroxyurea was mediated by the inhibition of the action of ribonucleotide reductase in deoxyribonucleotide synthesis, measurements were made of intracellular contents of dNTPs during the inhibition of cell growth by 1 mM hydroxyurea (Table 3). The most marked effect of hydroxyurea treatment was a 42% decrease in dATP content over 20 min. This result is consistent with the functioning of a ribonucleoside diphosphate reductase inhibited by hydroxyurea and having dATP as a negative effector. Thus, when the enzyme is partially inhibited by hydroxyurea, the inhibition will be compensated to some degree by the attenuation of the negative feedback through the depletion of dATP.

TABLE 3. Effect of hydroxyurea on the deoxynucleoside triphosphate contents of *M. mycoides*

Nucleotide	Nucleotide content (nmol/g of protein) at time (min)[a]			
	−4	−2	5	20
dGTP	40	40	44	58
dATP	106	102	78	60
dCTP	70	68	50	60
dTTP	132	116	110	104

[a] Hydroxyurea to 1 mM was added at zero time.

DISCUSSION

The results on the incorporation of precursors into DNA in conjunction with our previous findings in relation to ribonucleotide metabolism (5–7) suggest that *M. mycoides* subsp. *mycoides* is able to synthesize pyrimidine deoxyribonucleotides via the reactions outlined in Fig. 1. Results from experiments on the incorporation of label from [*U*-^{14}C]cytidine show that cytidine, in the presence of uracil and thymine, provided all of the dCMP in *M. mycoides* DNA. These results also provide evidence for a salvage synthesis of dTMP from thymine via thymidine phosphorylase using deoxyribose-1-phosphate derived from cytidine. The exclusion of [^{14}C]cytidine label from [^{32}P]dTMP was greatest with dUMP and dCMP, consistent with the proposed intracellular deamination of dCMP to dUMP with the subsequent release of the deoxyribose moiety for dTMP synthesis. The exclusion of the [*U*-^{14}C]cytidine label by deoxycytidine and dCMP was greater from DNA thymidylate than from DNA deoxycytidylate; this was consistent with the pathway proceeding via the deamination of dCMP, and not via that of dCTP, as occurs in enterobacteria (9, 10). The direct use of exogenous dTMP without prior dephosphorylation provided most of the dTMP in DNA. This direct use of dTMP and of the other dNMPs has only been observed previously for *Bdellovibrio bacteriovorus*, which grows intraperiplasmically in *E. coli* (14). Possibly, a mechanism for such direct uptake may occur more generally in organisms which grow parasitically in association with host cells.

We did not observe rNMP incorporation into ribonucleotides isolated from RNA (data not shown); yet, there was evidence for the uptake of rNMP intact. AMP, CMP, and GMP partially excluded $^{32}P_i$ from their respective dNMPs. This might indicate the uptake of rNMP and metabolism to the diphosphate level, followed by reduction, to yield the deoxynucleotide precursor for the synthesis of DNA, without significant utilization as rNTP for stable RNA synthesis.

Our data also showed that *M. mycoides* uses the phosphate moiety from exogenous dNMP in competition with the large excess of $^{32}P_i$ in the growth medium, providing evidence for intracellular dNMP phosphatase activity. The least effective dNMP as a source of phosphate to dilute the $^{32}P_i$ incorporated into dCMP in DNA was dAMP, indicating that the cells may take up dAMP less effectively than the other dNMPs. The use of ribonucleosides or ribonucleotides as precursors of dNMPs and the effects of hydroxyurea on cell growth and levels of dNTP are consistent with *M. mycoides* having a ribonucleoside diphosphate reductase with some properties similar to those of the *E. coli* enzyme.

The previous finding that cytidine in the presence of uracil gives rise to about 20% of RNA uridylate (5) can be interpreted in terms of the reaction sequence from cytidine to uracil and then RNA (Fig. 1). Our observation that cytidine can replace uracil for cell growth in media containing thymine suggests that cytidine may meet the requirement for uridine nucleotides via the reaction sequence shown in Fig. 1 if, as reported previously, the organism has no cytidine deaminase. However, the incorporation of cytidine ribose into UMP is approximately threefold greater than into AMP or GMP, suggesting that the deamination of cytidine may also occur at the ribonucleoside or ribonucleotide level.

There are at least three possible routes by which pentose carbon from cytidine could enter purine dNMP and rNMP. These include the formation of purine dNMP from dRib-1-P (Fig. 1) via the action of a deoxyribonucleoside phosphorylase and a kinase; the metabolism of dRib-

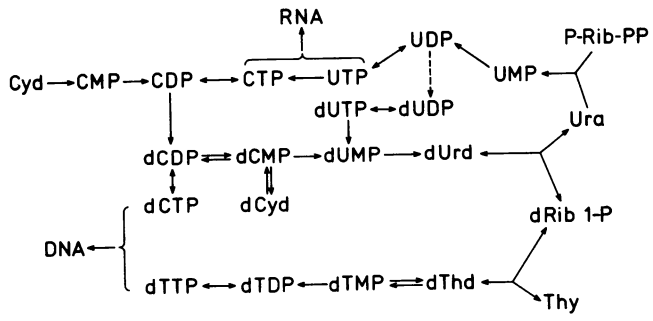

FIG. 1. Proposed pathways for pyrimidine deoxyribonucleotide synthesis in *M. mycoides* subsp. *mycoides*. Symbols: →, direction of reaction consistent with observation; ↔ and ←⤹, reactions known to be effectively reversible in cells; ⇄, interconversion observed in both directions, not necessarily via a reversible reaction; and ⇢, possible reaction of limited significance.

1-P (Fig. 1) via glyceraldehyde 3-P and reactions of the pentose phosphate pathway to ribose-5-P and, thence, P-Rib-PP and purine ribonucleotides; and the metabolism of cytidine to P-Rib-PP at the ribonucleoside level, e.g., via the action of a cytidine deaminase and uridine phosphorylase. Our results do not exclude any of these possibilities.

Studies (manuscript in preparation) on enzymatic activities in extracts of *M. mycoides* largely substantiate the pathways outlined in Fig. 1.

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