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Genetic and Physiological Characterization of the Purine Salvage Pathway in the Archaebacterium *Methanobacterium thermoautotrophicum* Marburg

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The enzymes involved in the purine interconversion pathway of wild-type and purine analog-resistant strains of *Methanobacterium thermoautotrophicum* Marburg were assayed by radiometric and spectrophotometric methods. Wild-type cells incorporated labeled adenine, guanine, and hypoxanthine, whereas mutant strains varied in their ability to incorporate these bases. Adenine, guanine, hypoxanthine, and xanthine were activated by phosphoribosyltransferase activities present in wild-type cell extracts. Some mutant strains simultaneously lost the ability to convert both guanine and hypoxanthine to the respective nucleotide, suggesting that the same enzyme activates both bases. Adenosine, guanosine, and inosine phosphorylase activities were detected for the conversion of base to nucleoside. Adenine deaminase activity was detected at low levels. Guanine deaminase activity was not detected. Nucleoside kinase activities for the conversion of adenosine, guanosine, and inosine to the respective nucleotides were detected by a new assay. The nucleotide-interconverting enzymes AMP deaminase, succinyl-AMP synthetase, succinyl-AMP lyase, IMP dehydrogenase, and GMP synthetase were present in extracts; GMP reductase was not detected. The results indicate that this autotrophic methanogen has a complex system for the utilization of exogenous purines.

As the terminal organisms in many anaerobic food chains, the methanogens play a key role in biodegradation reactions. These organisms are phylogenetically diverse and metabolically rather limited, being capable of methanogenesis from only a few simple substrates (20, 21). They utilize novel coenzymes, lipids, cell walls, and metabolic reactions (3, 20, 21, 37). Molecular biology studies have revealed aspects of gene organization and regulation (6, 21) that support the inclusion of methanogens in the kingdom *Archaebacteria* and show their similarity to both eubacteria and eucaryotes. The genetic approach to the study of these organisms has yielded mutants and physiological information, but genetic exchange mechanisms are in the very early stages of development (4, 32, 45).

Although great strides have been made in understanding methanogens, little is known about the biosynthesis of nucleotides in these organisms. Purine nucleotides can be synthesized from simple precursors by de novo pathways; however, salvage of preformed bases and nucleosides can provide an energy-efficient alternative. Salvage pathways have been found in eucaryotes (1, 13, 16) and eubacteria (12, 43) and appear to be present in archaebacteria (5, 23, 33, 42). Knowledge of purine salvage pathways in methanogens is important because it increases our knowledge of methanogen metabolism, which can be applied to the selection and use of radioactive compounds for labeling nucleic acids and their precursors. It provides insight and understanding of the mechanism of action of purine analogs and allows for the isolation of purine analog-resistant mutants blocked in various steps of the salvage pathways. Because methanogens are resistant to many of the more common antibiotics, purine analogs have been valuable tools in genetic and physiological studies. Mutant strains resistant to purine analogs have been isolated from *Methanococcus voltae* (5), *Methanobacterium thermoautotrophicum* Marburg (45), *Methanobacterium* sp. strain FR-2, *M. bryantii* Mo.H.G., *M. smithii* PS, and *Methanosarcina mazei* S-6 (23). The thermophilic autotroph *M. thermoautotrophicum* was selected for this study because it is biochemically well characterized. It contains a cryptic plasmid (30) and can be infected by a bacteriophage (29); various mutants have been collected (22, 33, 36); and a genetic transformation system has been described (45).

In this report, we provide a characterization of the network of purine base, nucleoside, and nucleotide interconversion reactions in this organism by means of labeling, mutant characterization, and enzymatic assays.

(A preliminary report of some of these results has been presented [V. E. Worrell and D. P. Nagle, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, I22, p. 221].)

MATERIALS AND METHODS

Abbreviations. $PRPP_i$, Phosphoribosylpyrophosphate; zn_2Pur, 8-aza-2,6-diaminopurine; sGua, 6-thioguanine; Shy, 6-mercaptopurine; zGua, 8-azaguanine; zHyp, 8-azahypoxanthine; n_2Pur, 2,6-diaminopurine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Materials. Natural bases, nucleosides, nucleotides, analogs, and $PRPP_i$ were purchased from Sigma Chemical Co., St. Louis, Mo. Gelrite gellan gum was obtained from Scott Laboratories, Fiskville, R.I. Noble agar was from Difco Laboratories, Detroit, Mich. [8-^{14}C]adenine (55 mCi/mmol) and [8-^{14}C]hypoxanthine (56 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. Omnifluor, [γ-^{32}P]ATP (3,000 Ci/mmol), and [8-^{14}C]guanine (40 to 60 mCi/mmol) were from Du Pont, NEN Research Products, Boston, Mass. Ribose-1-phosphate was from Aldrich Chemical Co., Inc., Milwaukee, Wis. Polyethyleneimine-cellulose thin-layer plates were from EM Science, Cherry Hill, N.J.

Bacterial strains and culture conditions. Table 1 lists the strains of *M. thermoautotrophicum* used in this study. Cultures were grown in liquid medium 2 (3) with shaking or were plated on solid mineral medium 2 in pressure vessels

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TABLE 1. Strains of *M. thermoautotrophicum* used in this study

Strain	Proposed genotype[a]	Phenotype selected	Original strain, source, or reference[b]
Marburg	Wild type	None	DSM 2133
RTAE-1	*fus-1*	Fluorouracil resistant	Reference 33
RTVW-1	*fus-1 par-1*	Fluorouracil resistant, mercaptopurine resistant	RTAE-1 (45)
VW-102	*par-2*	Azahypoxanthine resistant	This work
VW-106	*par-3*	Mercaptopurine resistant	This work
VW-107	*ahar-1*	8-Aza-2,6-diaminopurine resistant	This work
VW-108	*par-4*	Azaguanine resistant	This work
VW-109	*par-5*	Thioguanine resistant	This work

[a] *fus*, Fluorouracil sensitive; *par*, purine analog resistant; *ahar*, adenine, hypoxanthine analog resistant.
[b] All mutant strains except RTVW-1 are derived from Marburg.

under an atmosphere of H_2-CO_2-H_2S (80:19:1, vol/vol/vol) at 200 to 300 kPa and 60°C (45). Solid medium contained 8 g of Gelrite gellan gum per liter or 20 g of agar per liter.

Inhibition by analogs. The inhibitory effects of each analog on both wild-type and mutant strains in liquid culture were studied. Sterile, anoxic solutions of the analog were added to mineral medium at a final concentration of 300 μg/ml. The degree of inhibition of growth was quantitated after 48 h by measurement of the A_{660} of duplicate tubes. Tubes without any of the analogs served as control. Each tube was repressurized with H_2-CO_2 at 24 h of incubation.

To determine the nature of inhibition, we added 500 μg of analog per ml to exponentially growing cultures and incubated the mixture for 18 h at 60°C. Each culture was diluted, spread on Gelrite plates without analog, and incubated for 5 days as described above. Colonies were counted to determine the number of viable cells in analog-treated and control cultures.

Rate of spontaneous mutation and selection of mutants. Spontaneously resistant mutants were isolated, and the rate of spontaneous mutations was estimated from three cultures by plating approximately 10^7 cells per plate on duplicate plates containing 100 μg of either zn_2Pur, zGua, or sGua per ml or 1 mg of zHyp or Shy per ml. Colonies were counted after 5 days of incubation, transferred, and maintained in liquid medium containing the appropriate analog. A wild-type culture was mutagenized by the addition of 100 μg of *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine per ml. The culture was pressurized and incubated at 60°C for 18 h. The cells were washed twice by centrifugation at 7,000 × *g* for 20 min and resuspension in fresh medium. The suspension was incubated for 18 h at 30 kPa. Approximately 10^8 cells were spread on duplicate plates containing 500 μg of zn_2Pur per ml. One colony grew and was subcultured as described above. Mutant strains were transferred repeatedly in medium 2 containing the appropriate analog, in mineral medium alone, and again in medium 2 containing the analog to ensure the stability of resistance. No strains used in this study were prone to reversion to the wild-type phenotype, and all were considered to be stable.

Antagonism of inhibitory compounds. Cells (10^7 to 10^8) were spread on agar plates containing 500 μg of analog per ml. Filter paper disks (diameter, 12.7 mm; Schleicher & Schuell, Inc., Keene, N.H.) containing 250 μg of the natural base or 500 μg of Casamino Acids (Difco) were placed on the plates. The plates were incubated for 5 days and the zones of growth around the disks were measured (in millimeters). Control disks contained 10 μl of anoxic, sterile water.

Preparation of cell extracts. Cultures (250 ml) of the various strains were grown with 100 μg of the appropriate purine analog per ml in medium 2 for 3 days in H_2-CO_2 at 20 kPa and harvested by centrifugation at 17,000 × *g* for 20 min at 4°C. Pellets were washed twice by suspension in 1 culture volume of sterile medium without resazurin or sulfide followed by centrifugation. They were used immediately or stored at −20°C with no loss of enzyme activity. Extracts were prepared aerobically. Cells (1 g [wet wt]) were suspended in 2 ml of buffer (50 mM Tris hydrochloride [pH 7.4], 2 mM $MgCl_2$, 7 mM 2-mercaptoethanol) and broken by passage through a French pressure cell (40 MPa). The lysate was centrifuged at 30,000 × *g* for 10 min at 4°C. The supernatant crude extract was used for all enzyme assays. A portion of crude extract was dialyzed at 4°C for 18 h against one change of 1,000 volumes of buffer and stored at −20°C. Dialysis did not significantly change the activity of the enzymes tested (adenine phosphoribosyltransferase, succinyl-AMP synthetase, AMP deaminase, and GMP reductase). Protein was determined by the method of Lowry et al. (25) with bovine serum albumin as the standard.

Enzyme assays. Phosphoribosyltransferase and nucleoside phosphorylase activities for adenine, guanine, and hypoxanthine and adenine and guanine deaminase activities were assayed by the conversion of radiolabeled base to product (43). The xanthine oxidase activity was assayed by using the same reaction mixture as that used for adenine deaminase, except that hypoxanthine was substituted for adenine. These assay mixtures were incubated at 50°C. Nucleoside kinase activities were assayed by a new method involving the formation of radioactive nucleotide from the nucleoside and [γ-^{32}P]ATP. The reaction mixture contained 50 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 30 μM nucleoside, [γ-^{32}P]ATP (0.174 μCi/μmol), 7.6 mM ATP, 0.5 mM KCl, and enzyme (15 to 30 μg of protein). The reaction mixture (2 μl) was spotted on thin-layer chromatography plates together with 20 nmol each of the respective unlabeled base, nucleoside, and nucleotide.

Radiolabeled substrate and product were separated by thin-layer chromatography on polyethyleneimine-cellulose plates. Bases were separated from nucleosides and nucleotides by a 1 M LiCl solvent system (43). XMP, IMP, ATP, and P_i were separated from each other by using an acetone–15% trichloroacetic acid (65:35) solvent system (7). AMP, ATP, and P_i were separated from each other by using an acetone–35% formic acid (6:4) solvent system (7). A solvent system consisting of acetone, 35% formic acid, and 15% trichloroacetic acid (6:4:1) was devised to separate GMP, ATP, and P_i, yielding R_f values of 0.48, 0.0, and 0.32, respectively. The areas containing purine bases and their derivatives were visualized under UV illumination, whereas phosphate was visualized by the molybdate-stannous chloride method of Stahl (41). For quantitation of the radiolabel, the spots containing base, nucleoside, and nucleotide were cut out and placed into scintillation vials containing 0.25 ml of water and 2.5 ml of Omnifluor scintillation cocktail. Radioactivity in samples was counted in a liquid scintillation counter (model LS 1701; Beckman Instruments, Inc., Fullerton, Calif.). Recovery of radioactivity spotted on thin-layer chromatography plates ranged from 95 to 110% for ^{14}C and 91 to 112% for ^{32}P. Radioactivities were determined by using the H number calculation.

Adenosine deaminase (34), AMP deaminase (9), IMP dehydrogenase (27), GMP synthetase (38), GMP reductase (26), succinyl-AMP synthetase (14), and succinyl-AMP lyase

TABLE 2. Inhibition of growth of *M. thermoautotrophicum* by purine analogs

Type of analog	Analog used	Growth (10^8 CFU/ml)
Control	None (H_2-CO_2)	6.8
	None (N_2-CO_2)	0.13
	None (bacitracin)	0.00009
Noninhibitory[a]	2-Amino-6-methylmercaptopurine	6.4
Inhibitory[b]	2,6-Diaminopurine	0.35
Bacteriostatic[c]	6-Methylaminopurine	0.09
	6-Methylmercaptopurine riboside	0.16
Bacteriocidal[d]	8-Aza-2,6-diaminopurine	0.0011
	6-Thioguanine	0.0004
	8-Azaguanine	0.0004
	6-Mercaptopurine	0
	8-Azahypoxanthine	0

[a] Same growth as H_2-CO_2 control. This class of compounds also includes 8-azaxanthine, 6-chloroxanthine, 6-chloropurine, 6-dimethylaminopurine, and isopropylidine guanoside.
[b] Less than H_2-CO_2 control, but greater than N_2-CO_2 control.
[c] Same as N_2-CO_2 control.
[d] Less than N_2-CO_2 control.

(44) activities were determined spectophotometrically at 55°C.

Xanthine phosphoribosyltransferase and xanthosine phosphorylase were assayed (2) at 60°C by using the orcinol reaction to indicate the amount of $PRPP_i$ or ribose-1-phosphate remaining (15).

Uptake of ^{14}C-labeled compounds. Incorporation of labeled bases was determined by the method of Bowen and Whitman (5), with the following modifications. The cultures were grown to stationary phase in mineral medium containing 0.1 mM adenine, guanine, or hypoxanthine and 0.05 μCi of the appropriate ^{14}C-labeled base per ml. A 0.2-ml sample was removed to determine the total amount of radiolabel present. A 1.0-ml portion of the culture was centrifuged at 14,000 × *g* for 90 s, and 0.2 ml of the supernatant was removed. The pellet was washed twice by suspension in fresh mineral medium without resazurin or sulfide added, followed by centrifugation. Samples (0.15 ml) of the whole culture and the resuspended, washed cell pellet were added to 0.1 ml of 10 mM NaOH and heated to 100°C for 10 min. The treated sample (0.2 ml) was placed in 2.5 ml of Omnifluor scintillation cocktail, and radioactivity was determined as described above.

RESULTS

Inhibitor sensitivities. Various purine nucleobase and nucleoside compounds were tested as possible inhibitors of the growth of *M. thermoautotrophicum*. Five base analogs, 2-amino-6-methylmercaptopurine, 8-azaxanthine, 6-chloroxanthine, 6-chloropurine, and 6-dimethylaminopurine, and one nucleoside analog, isopropylidine guanoside, did not inhibit the growth of *M. thermoautotrophicum* when they were added at concentrations up to 1.0 mg/ml. Eight analogs affected the growth of the organism. Two of these were bacteriostatic, one was inhibitory, and five were strongly bacteriocidal (Table 2). The rate of spontaneous mutation was estimated for the analogs which were bacteriocidal: zn_2Pur, $<8.5 \times 10^{-9}$; zGua, 4.4×10^{-7}; sGua, 1.7×10^{-6}; zHyp, 1.2×10^{-6}; Shy, 2.8×10^{-9}.

Isolation and characterization of mutants. All but one of the analog-resistant strains were spontaneous isolates. Spontaneous resistance of the wild-type culture to zn_2Pur was never observed, but after mutagenesis a single resistant strain (VW-107) was isolated.

TABLE 3. Growth of *M. thermoautotrophicum* strains in the presence of purine analogs

Strain	Growth (%) of strain in presence of[a]:				
	8-Aza-2,6-diamino-purine	8-Azahypo-xanthine	6-Mercapto-purine	8-Aza-guanine	6-Thio-guanine
Wild type	0	0	0	0	0
VW-102	18	100	43	98	75
VW-106	51	98	104	93	69
VW-107	100	111	0	0	0
VW-108	26	82	86	83	86
VW-109	29	94	91	108	91
RTVW-1	12	117	55	91	98

[a] Growth is expressed as a percentage of that in untreated control culture (A_{660}, 0.3 to 0.6). Values are the averages of duplicate determinations. All analogs were present at 300 μg/ml.

The susceptibility of each mutant strain to the inhibitory effects of the other purine analogs was determined (Table 3). Three hypoxanthine analog-resistant strains, VW-102 ($zHyp^r$), VW-106 (Shy^r), and RTVW-1 (Shy^r, 5-fluorouracil resistant), were also resistant to the guanine analogs zGua and sGua. The two guanine analog-resistant mutants, VW-108 ($zGua^r$) and VW-109 ($sGua^r$), also grew in the presence of the hypoxanthine analogs. All mutants showed an increased tolerance for zn_2Pur and zHyp compared with the wild type, although, since the last two analogs are bacteriocidal for the wild type, the decreased levels of growth could be a result of slow killing by the analog. Strain VW-107 (zn_2Pur^r) was cross-resistant only to zHyp.

Shy^r mutant strains (VW-106 and RTVW-1) differed morphologically from the wild type in liquid culture. Cultures of these two strains grew in clumps which appeared long and stringy after vortexing. These cultures produced methane, and on microscopic examination, cells resembled the wild-type cells but formed long chains. These cultures also lacked the characteristic yellow-green fluorescence of the deazaflavin compounds secreted by *M. thermoautotrophicum*.

Antagonism of inhibitors. The ability of natural bases or Casamino Acids to reverse the inhibition by the purine analogs was determined in an effort to understand purine base and nucleoside salvage. As determined on solid medium, neither adenine nor Casamino Acids spared any cultures from inhibition by the bacteriocidal analogs. Both guanine and hypoxanthine permitted the growth of *M. thermoautotrophicum* in the presence of Shy or sGua, although hypoxanthine was the more potent antagonist as measured by the diameter of growth surrounding the disk containing it (results not shown). The inhibitory effects of zHyp and methyl mercaptopurine riboside were countered only by the addition of hypoxanthine. The inhibition of growth by zn_2Pur, n_2Pur, or zGua was not reversed by any addition.

Uptake of natural bases. The ability of the wild-type and mutant strains to incorporate natural bases into cell material during growth was measured (Table 4). The wild-type strain of *M. thermoautotrophicum* incorporated adenine, guanine, and hypoxanthine from the media. Strains VW-102, VW-106, VW-108, and VW-109 incorporated as much adenine as did the wild-type strain, but the uptake of guanine and hypoxanthine was only 0.2 to 2% of that of wild type. The uptake of guanine and hypoxanthine in the doubly marked strain RTVW-1, resistant to both Shy and 5-fluorouracil, was similar to that of the other mutants. However, RTVW-1

TABLE 4. Incorporation of ^{14}C-labeled purines by *M. thermoautotrophicum* strains[a]

Strain	Amt (nmol) of base incorporated/10^8 cells		
	Adenine	Guanine	Hypoxanthine
Wild type	11	29	12
VW-102	17	0.31	0.19
VW-106	17	0.10	0.04
VW-107	0.37	6.3	0.07
VW-108	16	0.44	0.11
VW-109	22	0.06	0.35
RTVW-1	2	0.84	0.27

[a] Cultures were grown to stationary phase in mineral medium containing 0.05 μCi of ^{14}C-labeled base per ml.

incorporated only 18% as much adenine as did wild-type cells. Strain VW-107 showed a different uptake pattern, incorporating 22% of the guanine that the wild type did, but less than 3% of the amount of adenine or hypoxanthine.

Enzyme activities. Cell extracts of wild-type cells were prepared to determine whether purine bases were activated to the nucleoside or nucleotide levels and interconverted. Hypoxanthine and guanine phosphoribosyltransferase activities were 1 order of magnitude greater than adenine and xanthine phosphoribosyltransferase activities (Table 5). The levels of salvage activities detected were of the order of magnitude required for the incorporation of bases observed. Adenosine phosphorylase activity was 5- to 10-fold greater than guanosine or inosine phosphorylase activities. Xanthosine phosphorylase activity was detected only at low levels. Activities for the interconversion of purine bases, adenine deaminase and xanthine oxidase, were low, whereas guanine deaminase was not detected. At the nucleoside level, adenosine deaminase and adenosine, guanosine, and inosine kinases were present at high levels; xanthosine kinase activity was not detected. The nucleotide-interconverting enzymes (i.e., AMP deaminase, IMP dehydrogenase, GMP synthetase, succinyl-AMP synthetase, and succinyl-AMP lyase activities) were found at significant levels. GMP reductase was not detected.

TABLE 5. Purine interconversion enzyme activities in extracts of *M. thermoautotrophicum* Marburg

Enzyme	Sp act (nmol of product/mg of protein per min)[a]
Phosphoribosyltransferases	
Adenine	0.14
Guanine	3.70
Hypoxanthine	1.37
Xanthine	0.06
Nucleoside phosphorylases	
Adenosine	0.20
Guanosine	0.04
Inosine	0.02
Xanthosine	<0.001
Nucleoside kinases	
Adenosine	7.50
Guanosine	37.5
Inosine	38.5
Xanthosine	<0.001
Deaminases	
Adenine	0.02
Adenosine	26.5
AMP	3.16
Guanine	<0.001
Xanthine oxidase	0.014
IMP dehydrogenase	1.99
GMP synthetase	3.84
GMP reductase	<0.001
Succinyl-AMP synthetase	28.3
Succinyl-AMP lyase	30.3

[a] Enzyme activities are the average of triplicate determinations of two different batches of extract.

TABLE 6. Enzyme activities of *M. thermoautotrophicum* strains

Strain	Amt of product[a] (nmol/mg of protein in 60 min)							
	Phosphoribosyl-transferase			Purine nucleoside phosphorylase			Deaminase	
	ADE	GUA	HYP	ADE	GUA	HYP	ADE	GUA
Wild type	5.0	66.4	51.6	6.0	0.4	0.3	0.9	0.4
VW-102	2.2	1.0	1.3	3.7	0.4	1.2	0.6	0.1
VW-106	7.6	1.7	117.2	9.3	1.1	0.0	0.8	0.9
VW-107	0.3	1.4	0.1	6.2	6.8	0.5	0.3	0.6
VW-108	8.0	0.8	0.7	7.8	1.1	0.1	4.4	0.3
VW-109	4.9	1.6	1.2	5.5	1.2	0.1	1.9	0.5
RTVW-1	2.2	0.3	1.9	2.7	1.3	0.1	1.8	1.0

[a] Abbreviations: ADE, adenine; GUA, guanine; HYP, hypoxanthine.

The enzymatic conversion of substrate to product was proportional to time under conditions of excess substrate and limiting enzyme. The specific activities (in nanomoles of product per milligram of protein per minute) were calculated from this linear region. Specific activities in extracts of the mutant strains are presented as nanomoles of product per milligram of protein per hour. Phosphoribosyltransferase activities were $PRPP_i$ dependent, whereas nucleoside phosphorylase activities were ribose-1-phosphate dependent. Enzyme activity was proportional to enzyme concentration from 15 μg to 1 mg of protein per ml of reaction. Three different batches of extract were tested, and *Escherichia coli* controls were run for the following enzyme activities (data not shown): xanthine phosphoribosytransferase; xanthine oxidase; adenosine, guanosine, inosine, and xanthosine kinases and phosphorylases; and guanosine reductase. Activities for xanthine oxidase, adenosine and xanthosine kinases, and xanthosine phosphorylase were undetected in *E. coli*.

Characterization of the pathway in mutant strains. Base analogs typically inhibit cell growth by being converted to nucleotides which interfere with metabolism or are incorporated into nucleic acids which are then defective in function. Resistance may be acquired by loss of the ability to incorporate the analog or the ability to metabolize the analog further. To determine whether the analog-resistant strains were altered in their ability to metabolize base derivatives, we prepared extracts of the mutant strains and tested them for the presence of the salvage enzymes (Table 6). These data might also help to explain the patterns of inhibition of growth by the various purine antimetabolites. In 1 h, extracts of strain VW-106 converted twice as much hypoxanthine to IMP as did the wild type, but much lower activity for the conversion of guanine to GMP was detected. The other five strains were deficient in both hypoxanthine and guanine phosphoribosyltransferase activities, converting less than 4% of the amount of base to the nucleotide in 1 h than did the wild-type strain. Adenine phosphoribosyltransferase activity was 17-fold lower in strain VW-107 and 2-fold lower in strains VW-102 and RTVW-1 than in the wild type. The activity of adenine deaminase in strain VW-108 (interconversion of adenine and hypoxanthine) was five times the level of

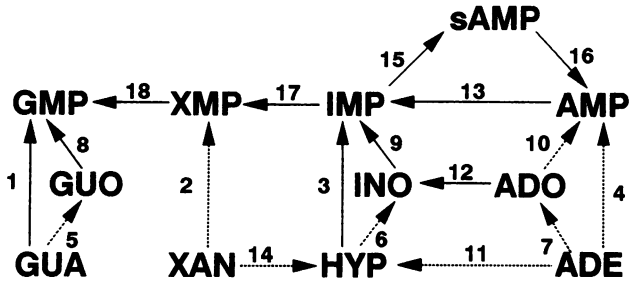

FIG. 1. Proposed pathway for purine interconversion in *M. thermoautotrophicum* Marburg. Symbols: ——►, activities greater than 1.0 nmol/mg of protein per min; ┈┈►, activities greater than 0.01 but less than or equal to 0.20 nmol/mg of protein per min. Reactions 1, 2, 3, and 4 represent guanine (GUA), xanthine (XAN), hypoxanthine (HYP), and adenine (ADE) phosphoribosyltransferases, respectively; 5, 6, and 7 represent guanosine (GUO), inosine (INO), and adenosine (ADO) nucleoside phosphorylases, respectively; 8, 9, and 10 represent GUO, INO, and ADO kinases, respectively; 11, 12, and 13 represent ADE, ADO, and AMP deaminases, respectively; 14 represents XAN oxidase; 15 represents succinyl-AMP (sAMP) synthetase; 16 represents sAMP lyase; 17 represents IMP dehydrogenase; and 18 represents GMP synthetase.

the wild-type and other strains. Conversion of purine bases to nucleosides by extracts from the mutants was different from that in the wild type in two cases: adenosine phosphorylase activity was twofold lower in strain RTVW-1 than in the wild type, and guanosine phosphorylase activity was 17-fold higher in strain VW-107 (obtained by mutagenesis) than in the wild type.

DISCUSSION

Pathways for interconversion of purine and purine derivatives have been found in eubacteria such as *E. coli* (35), *Bacillus subtilis* (12, 39), and spirochetes (8) and in eucaryotes such as parasitic protozoa (1), fungi (16), and mammals (31). The results of our work demonstrate a purine salvage and interconversion pathway for the autotrophic methanogen *M. thermoautotrophicum* (Fig. 1). The rationale for this proposed pathway is outlined below.

M. thermoautotrophicum is susceptible to seven purine analogs, five of which are bacteriocidal, and to one nucleoside analog, which is bacteriostatic. This suggests that *M. thermoautotrophicum* can activate bases and nucleosides to nucleotides. Noninhibitory analogs may not be adequately transported or activated or may be metabolized (e.g., by dechlorination of chloroxanthine).

Labeling experiments with wild-type *M. thermoautotrophicum* suggest that adenine, guanine, and hypoxanthine are incorporated into cell material and provide further evidence for the activation of natural bases. The amounts of purines incorporated per cell were somewhat higher than those reported for *Methanococcus voltae* (5) or for the incorporation of uracil by *M. thermoautotrophicum* (33). Knox and Harris (23) reported that two mesophilic *Methanobacterium* spp. were inhibited by Shy and the nucleoside analog 7-deazaadenosine and that *Methanobacterium* sp. strain FR-2 was inhibited by zGua, Shy, and 7-aza-adenosine. These results indicate that purine base and nucleoside utilization may be widespread in this genus. Members of the methanogen genera *Methanobrevibacter*, *Methanococcus*, and *Methanosarcina* are also inhibited by purine analogs (5, 23). *Methanococcus vannielii* can degrade purines and utilize them as the sole source of nitrogen (10, 11).

The detection of purine phosphoribosyltransferase activities in extracts of wild-type cells (Table 5) shows that a mechanism for activation of exogenous adenine, guanine, hypoxanthine, and xanthine exists in *M. thermoautotrophicum* and is the major rationale for the reactions from base to nucleotide (Fig. 1). There is little activity in extracts to catalyze the conversion of one purine base to another (Fig. 1; Table 5). Adenine was the only base converted to the nucleoside at significant levels. Kinase activities for the conversion of adenosine, guanosine, and inosine to their respective nucleotides were detected in cell extracts (Fig. 1; Table 5). Activity for the conversion of adenosine to inosine (adenosine deaminase) was highly active. If the in vitro levels of enzymes detected reflect the in vivo condition, the predominant pathway for metabolism of adenosine may be through inosine to IMP, because the specific activity of adenosine deaminase was 3.5 times that of adenosine kinase.

The pathway is completed by the reactions of the purine nucleotide interconversion shown in the figure. IMP was converted to AMP in two steps, succinyl-AMP synthesis and hydrolysis; AMP was deaminated to IMP by AMP deaminase. IMP dehydrogenase catalyzed the formation of AMP from IMP, and GMP synthetase formed GMP from XMP. Activity for GMP reductase was not detected. Indirect evidence for the lack of this enzyme is also provided by the facts that (i) guanine did not protect cells from the adenine analog zn_2Pur; (ii) hypoxanthine was more effective at sparing cells from the guanine and hypoxanthine analogs than was guanine; and (iii) Rechsteiner et al. (36) reported an auxotrophic strain of *M. thermoautotrophicum* Marburg isolated after mutagenesis with a requirement for adenosine, which can grow with either adenine or hypoxanthine but not guanine. This suggests that both adenine and hypoxanthine, but not guanine, can be converted to adenosine or AMP.

Studies with purine analog-resistant mutants (Table 1) also support the pathway. Purine bases are thought to be transported into eubacterial cells by a group translocation process in which the base is converted to the nucleotide upon transport in the presence of $PRPP_i$ (18, 19). The results in this paper suggest that phosphoribosyltransferase activity is responsible for uptake in *M. thermoautotrophicum* as well. Phosphoribosyltransferase activity correlated with uptake activity in all strains except strain VW-106, which was deficient in guanine and hypoxanthine uptake but retained hypoxanthine phosphoribosyltransferase activity. A similar situation, the presence of phosphoribosyltransferase activity for a base accompanied by loss of uptake for that base, was described for *Salmonella typhimurium* Shy^r mutants (46). In this case, the specificity of the phosphoribosyltransferase activity for specific purine bases changes when the enzyme is released from the membrane (19). This, together with the altered morphology of the strain (suggesting an altered membrane), may explain the loss of hypoxanthine uptake with retention of hypoxanthine phosphoribosyltransferase activity in strain VW-106.

Hypoxanthine phosphoribosyltransferase activity is present in an enzyme distinct from those for guanine and xanthine in *E. coli* (17). A single enzyme converts hypoxanthine and guanine to their corresponding nucleotides in mammalian cells (13), yeasts such as *Saccharomyces cerevisiae* (1), and some bacteria such as *Lactobacillus casei* (24). On the basis of indirect evidence presented here, *M. thermoautotrophicum* appears to contain one enzyme capable of both hypoxanthine and guanine phosphoribosyltransferase activities, because, with the exception of strain VW-106 (see above), a loss of one activity resulted in a

concurrent loss of the other and both guanine and hypoxanthine protected cells from the analogs Shy and sGua.

Strain VW-107, obtained after mutagenesis, lacked adenine uptake and phosphoribosyltransferase activity, which is sufficient to explain its resistance to the adenine analog zn_2Pur. This strain retained adenosine phosphorylase activity (for conversion of adenine to adenosine), but was resistant to the adenine analog. This, along with the fact that adenosine phosphorylase was the only phosphorylase activity detected, suggests that adenosine phosphorylase may be used exclusively for endogenous salvage of adenine, and not for transport. In thermophiles, spontaneous depurination of DNA provides an internal source of purines needing reactivation. Strain VW-107 also lacked hypoxanthine uptake and guanine and hypoxanthine phosphoribosyltransferase activities. This strain was able to incorporate guanine; extracts activated it to the nucleoside (an activity detected only at low levels in the wild type). This explains the sensitivity of this strain to Shy, zGua, and sGua.

Strain VW-108 extracts contained high levels of adenine deaminase, an activity present only at low levels in the wild-type strain. In both VW-107 and VW-108, activities may be due to enzymes that were induced by growth in the presence of purine analogs. Since the wild-type strain was not grown in the presence of natural bases or analogs, these activities may not have been induced.

We have not considered pathways of deoxyribonucleoside and deoxyribonucleotide metabolism in *M. thermoautotrophicum*. It is known that this organism can form pyrimidine deoxyribonucleotides from ribonucleosides, since deoxycytidine is a precursor for DNA synthesis in vivo (40) and fluorodeoxyuridine is inhibitory to growth (42). Presumably, these deoxynucleosides are activated by a kinase activity, as shown for more familiar organisms (28, 35). Further investigation of such pathways will complement what is known about salvage and synthesis pathways in this versatile autotroph.

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