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Incorporation of Exogenous Purines and Pyrimidines by *Methanococcus voltae* and Isolation of Analog-Resistant Mutants

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Methanococcus voltae **incorporated exogenous adenine, guanine, hypoxanthine, and uracil, but not thymine. Growth of *M. voltae* was also sensitive to purine and pyrimidine analogs. Of the 20 analogs tested, 12 were inhibitory at 1 mg/ml. The most effective inhibitors were purine analogs with endocyclic substitutions. Nucleoside analogs and analogs with exocyclic substitutions or additions were less effective. Four purine analogs, 8-aza-2,6-diaminopurine, 8-azaguanine, 8-azahypoxanthine, and 6-mercaptopurine and one pyrimidine analog, 6-azauracil, were especially toxic. The MICs were 20, 0.5, 2.0, 80, and 10 μg/ml, respectively. Spontaneous resistance mutants were isolated for these five analogs. The MICs for these mutants were 20.5, 8.2, >65, >41, and 20.5 mg/ml, respectively. These concentrations far exceeded the solubilities of the analogs and represented an increase in resistance of at least three orders of magnitude. In addition to demonstrating cross resistance to several of the analogs, four of these mutants lost the ability to incorporate exogenous bases. These appeared to be mutations in the salvage pathways for purines and pyrimidines. In contrast, the mutant resistant to 6-mercaptopurine was not defective in purine uptake. Instead, it degraded 6-mercaptopurine. In the presence or absence of high concentrations of the analogs, the growth rates of the resistant mutants were no less than one-half of the growth rate of the wild type in the absence of the analog. The high level of resistance and rapid growth are very desirable properties for the application of the mutants in genetic experiments.**

Methanogens are strictly anaerobic bacteria which rely on the production of methane from a limited number of simple carbon compounds as their sole source of energy. They are also archaebacteria and are as distantly related to eubacteria as they are to eucaryotes (35). Thus, methanogens represent a biochemically unique group of bacteria, and the study of their genetics is essential to our understanding of these microorganisms.

Methanococcus voltae has a number of properties that are ideal for genetic studies. It is the fastest growing methanogen at mesophilic temperatures, and it has a doubling time of 90 min under optimal conditions. Its nutritional requirements have been well characterized (33). It has a single-cell morphology and a high plating efficiency (16). It has a protein cell wall, which facilitates the purification of plasmid and chromosomal DNA. Also, a number of closely related isolates have been obtained (34). One strain of *Methanococcus* sp. has been found to harbor a small plasmid which may provide the basis for a cloning vector (37). Most importantly, *M. voltae* is the only methanogen in which transformation has been demonstrated (G. Bertani, Genetics **113**:s74, 1986). These developments are the first steps toward a genetic system in methanogens.

Although several genes from *Methanococcus* sp. and other methanogens have been cloned into eubacteria and subsequently sequenced (1, 2, 5, 10, 11, 19, 23, 26, 29, 31, 36), little is known concerning genetic transfer within this group of organisms. To complicate matters, methanococci are resistant to many antibiotics which are routinely used in genetic manipulations (8, 14, 24). Until recently, no mutants were available to serve as genetic markers for the development of a genetic system. To date, only seven mutants of *M. voltae* have been reported. Two are auxotrophs which require either histidine or purines (G. Bertani and L. Baresi, Abstr. European Molecular Biology Organization Workshop on the Molecular Genetics of Archaebacteria, 1985, A1). The remainder are mutants that are resistant to low concentrations of the amino acid analogs azaserine, methionine sulfoximine, and 5-methyltryptophan, and the coenzyme M analog bromoethanesulfonic acid (28; P. Gernhardt and A. Klien, Abstr. European Molecular Biology Organization Workshop on the Molecular Genetics of Archaebacteria, 1985, B3).

In this report we demonstrate the incorporation of exogenous purines and pyrimidines by *M. voltae* and the presence of a salvage pathway. Metabolism of these bases has also been demonstrated in *Methanococcus vannielii* and *Methanobacterium thermoautotrophicum* (3, 7, 32). Therefore, the sensitivity of *M. voltae* to base analogs was examined to characterize the nature of this incorporation and to obtain additional selective agents for the isolation of mutants. Of the 20 analogs tested, 12 were inhibitory. Resistance mutants were isolated for five of these analogs. Mutants of methanogens resistant to one of these analogs, 6-mercaptopurine (Shy), have previously been isolated in the genera *Methanobrevibacter* and *Methanobacterium* (J. E. Harris, D. M. Evans, and M. R. Knox, Abstr. European Molecular Biology Organization Workshop on the Molecular Genetics of Archaebacteria, 1985, B5). In addition, mutants of *Methanobacterium* sp. resistant to 5-fluorouracil have also been reported (32).

MATERIALS AND METHODS

Chemicals. Natural bases and base analogs were obtained from Sigma Chemical Co. (St. Louis, Mo.). [8-^{14}C]adenine, [8-^{14}C]hypoxanthine, [2-^{14}C]thymine, and [2-^{14}C]uracil were obtained from Research Products International Corp. (Mount Prospect, Ill.). [8-^{14}C]guanine was obtained from ICN Biochemicals Inc. (Irvine, Calif.). All other biochemicals were obtained from Sigma.

Culture conditions. *M. voltae* PS was grown in the complex medium described by Whitman et al. (33), with 0.2%

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TABLE 1. Incorporation of ^{14}C-labeled bases by the wild-type and mutant strains[a]

Base	μmol incorporated/g (dry wt) of cells by strains of the following phenotypes:					
	Wild type	Znp^r	Zgu^r	Zhy^r	Zur^r	Shy^r
Adenine	185	180	136	160	164	168
Hypoxanthine	175	182	0.5	0.3	140	195
Guanine	137	4.2	1.2	1.1	148	116
Uracil	117	134	149	179	0.12	181
Thymine	0.04	0.04	0.02	0.04	0.01	0.05

[a] Cultures were grown in defined medium supplemented with 0.05 μCi of ^{14}C-labeled base per ml. The incorporation was determined at the stationary phase, at which time no more than 80% of the total label added was incorporated.

vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) substituting for yeast extract. The cultures were incubated under an atmosphere of 40 lb/in^2 H_2-CO_2 (80:20; vol/vol) at a temperature of 30°C (33).

Incorporation. Cultures were grown to the stationary phase in defined medium (33) containing 0.1 mM adenine, guanine, hypoxanthine, thymine, or uracil and 0.05 μCi of ^{14}C-labeled base per ml. A 0.2-ml sample was removed to determine the total amount of radiolabel present. A portion of the culture was centrifuged at 15,000 × *g* for 90 s, and 0.2 ml of the supernatant was removed. The pellet was washed twice and suspended in an equal volume of Mc buffer (4.5 mM KCl, 13.5 mM $MgCl_2 \cdot 6H_2O$, 14.0 mM $MgSO_4 \cdot 7H_2O$, 9.0 mM NH_4Cl, 0.95 mM $CaCl_2 \cdot 2H_2O$, 0.8 mM K_2HPO_4, 0.38 M NaCl). The radioactivity in each sample, 0.2 ml, was determined by adding 0.1 ml of 10 mM NaOH and 5 ml of scintillation cocktail (2,330 ml of toluene, 1,165 ml of Triton X-100, 20 g of PPO [2,5-diphenyloxazole], 0.5 g of POPOP [1,4-bis(5-phenyloxazolyl)benzene]), followed by analysis in a liquid scintillation counter (type LS 3801; Beckman Instruments, Inc., Fullerton, Calif.). The percent incorporation of a base was defined as the disintegrations per minute in the pellet divided by the disintegrations per minute in the whole culture times 100. The micromoles of base incorporated was defined by the following formula: (disintegrations per minute of the pellet/disintegrations per minute of the whole culture) (micromoles of base added)/(grams [dry weight] of cell per culture). The remaining culture was sedimented, and the chromosomal DNA was purified by the procedure described by Wood et al. (36). ^{14}C-labeled whole cells were fractionated by the procedure described by Whitman et al. (33). The radioactivity was determined as described above.

Plating of *M. voltae*. Cultures were plated inside an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.), as described by Jones et al. (16), with the following modifications. The incubation canister was constructed from a 2-gallon (7.6-liter) pressure tank (model 83-5660; Binks Manufacturing Co., Franklin Park, Ill.) by the addition of a toggle valve for the admission of gas and a pressure gauge to monitor gas consumption during incubation. The canister was pressurized to 20 lb/in^2 with H_2. Instead of adding H_2S gas, H_2S was provided by the addition of 10 ml of a 20% Na_2S solution to the canister prior to pressurization. The canisters were removed from the glove box and incubated at 30°C for 5 to 10 days, depending on the experiment.

MICs. The analogs were added directly to sterile culture tubes. Sterile medium was inoculated with approximtely 10^5 cells per ml and added to the culture tubes inside an anaerobic glove box. The tubes were sealed and pressurized with H_2-CO_2. The MIC was defined as the lowest concentration of the analog which prevented growth for 3 days after growth of the control, which contained no analog.

RESULTS AND DISCUSSION

Incorporation of exogenous bases. The wild-type strain of *M. voltae* incorporated large amounts of guanine, adenine, hypoxanthine, and uracil (Table 1). However, thymine was not incorporated. The dry weight of *M. voltae* is 13 to 18% nucleic acid (33), and the amount incorporated fulfilled this base requirement. Fractionation of whole cells demonstrated that 78% of the radiolabel from [8-^{14}C]hypoxanthine was found in the hot trichloroacetic acid-soluble or nucleic acid fraction. No more than 10% was found in any other fraction (data not shown). This result indicates that exogenous bases are incorporated into nucleic acids by whole cells of *M. voltae* and are not degraded to a large extent, as occurs in *M. vannielii* (7). Analysis of purified chromosomal DNA indicated that 700 μmol of guanine was incorporated per g of DNA (data not shown). Based on a G+C content of 30 mol%, the chromosome was expected to contain approximately 450 μmol of guanine and 1,050 μmol of adenine per g of DNA (33). Therefore, *M. voltae* obtained about half of its purines in DNA from guanine in the medium, and guanine was converted to adenine. Incorporation of cytosine was not examined because none of the cytosine analogs tested were inhibitory (see below). In addition, uptake of thymine and cytosine is rare in bacteria (15). The incorporation of exog-

TABLE 2. Inhibition of *M. voltae* by base analogs[a]

Analog	CFU/ml (10^8)
Control	
None (H_2-CO_2)	3.5
None (N_2-CO_2)[b]	0.77
Noninhibitory[c]	
2-Thioxanthine	3.1
2-Mercaptopyrimidine	5.0
Inhibitory[d]	
2-Amino-6-methylmercaptopurine	2.3
8-Azaadenine	1.6
6-Azauridine	2.4
1-Methylthymine	2.7
6-Methyluracil	2.4
2-Thiouracil	1.8
Bacteriostatic[e]	
8-Aza-2,6-diaminopurine	0.76
6-Mercaptopurine	0.91
Bacteridical[f]	
8-Azaguanine	0.20
8-Azahypoxanthine	0.12
6-Azauracil	0.06

[a] A concentration of the analogs of 1 mg/ml was added to cultures during exponential growth and incubated for 12 h at 30°C. Viability was determined by plating each culture on complex medium.

[b] The culture was starved for H_2 to prevent growth with no significant loss of viability.

[c] Viability was the same or greater than that in the H_2 control. Also in this category were 5-azacytidine, 6-azacytosine, 4-hydroxy-6-methyl-2-thiopyrimidine, 5-hydroxymethyluracil, 2-thio-6-azauridine, and 6-mercaptoguanosine.

[d] Viability was less than that in the H_2 control but greater than that in the N_2 control. Also in this category was 6-azathymine.

[e] Viability was the same as that in the N_2 control.

[f] Viability was less than that in the N_2 control.

TABLE 3. MICs of the analogs for the wild type and mutants[a]

Phenotype	MIC (mg/ml) of the following analogs:				
	zn_2Pur	zGua	zHyp	Shy	zUra
Wild type	0.02	0.0005	0.002	0.08	0.002
Znp^r	20.5	0.016	0.25	0.16	0.002
Zgu^r	20.5	8.2	>65.5	>41.0	0.002
Zhy^r	20.5	8.2	>65.5	>41.0	0.002
Zur^r	0.02	0.004	0.001	0.08	20.5
Shy^r	20.5	2.1	>65.5	>41.0	0.002
Znp^r Zgu^r[b]	20.5	8.2	—[c]	—	—
Znp^r Zhy^r	20.5	—	>65.5	—	—
Znp^r Zur^r	20.5	—	—	—	20.5
Znp^r Shy^r	20.5	—	—	>41.0	—
Zgu^r Zur^r	—	2.1	—	—	20.5
Zhy^r Zur^r	—	—	>65.5	—	20.5
Zur^r Znp^r	20.5	—	—	—	20.5
Zur^r Zgu^r	—	2.1	—	—	20.5
Zur^r Zhy^r	—	—	>65.5	—	20.5
Zur^r Shy^r	—	—	—	>41.0	20.5
Shy^r Zur^r	—	—	—	>41.0	20.5

[a] MICs were determined in complex medium.
[b] Double mutants were generated from the single mutants by spontaneous mutation. The first phenotype denotes that of the parent strain.
[c] —, Not determined.

enous bases into nucleic acid demonstrates that there is a salvage pathway in this methanogen.

Sensitivity to purine and pyrimidine analogs. The effects of base analogs on the growth of *M. voltae* were examined to further characterize the incorporation of exogenous bases. Of the 20 purine and pyrimidine analogs that were examined, 12 inhibited the growth of *M. voltae* at concentrations of approximately 1 mg/ml. Of these 12, 2 were bacteriostatic, 3 were bactericidal, and the remaining 7 only partially inhibited growth (Table 2). The MICs of the bactericidal and bacteriostatic analogs were between 0.5 and 80 μg/ml (Table 3), which is within the range observed for other inhibitors of methanococci (14, 24, 28). These MICs were similar in both complex and defined media (data not shown). This result indicates that the Casamino Acids had no observable effect on the inhibitory activity of these analogs.

The analogs tested can be grouped into two categories: those which have exocyclic substitutions, such as additional amino, methyl, or hydroxyl groups, and those with endocyclic substitutions, namely, a nitrogen for a carbon. The endocyclic substitutions were generally more inhibitory. For example, the most potent analogs, which were either bacteriostatic or bactericidal, had a substitution of a nitrogen for either C-8 of the purines or C-6 of the pyrimidines. The analog Shy, which had an exocyclic substitution, was also very inhibitory. However, the MIC of Shy was at least four times higher than the MICs of the endocyclically substituted analogs (Table 3). Because inhibition by base analogs generally relies on their conversion to nucleotides (20), the enzymes of the salvage pathway in *M. voltae* may not efficiently utilize analogs with exocyclic substitutions. Therefore, these analogs would not be expected to be strongly inhibitory.

Although only four nucleoside analogs were tested (5-azacytidine, 2-thio-6-azauridine, 6-mercaptoguanosine, and 6-azauridine), they were generally less inhibitory than the base analogs. Only 6-azauridine was inhibitory at all, and it was much less so than the corresponding base analog 6-azauracil (zUra). This large difference in activity suggests that nucleosides are not taken up to the same extent as bases.

The analogs can be further subdivided according to the natural base which they most resemble. The guanine analog 8-azaguanine (zGua) was bactericidal. However, if the keto group at position C-6 was replaced by an amino group to give 8-aza-2,6-diaminopurine (zn_2Pur), the analog was bacteriostatic. Therefore, the amino group detracted from the activity of the aza group. zn_2Pur also resembled adenine. It was unlikely that zn_2Pur was an adenine analog, however, because 8-azaadenine (zAde) was only partially inhibitory and an exocyclic substitution would detract from its activity. 8-Azahypoxanthine (zHyp), a hypoxanthine analog, also had bactericidal activity. If the keto group of hypoxanthine was substituted by a mercapto group to give Shy, another hypoxanthine analog, the compound was bacteriostatic and less inhibitory than the aza analog. Therefore, the most inhibitory purine analogs were analogs of guanine and hypoxanthine.

Although more pyrimidine than purine analogs were examined, fewer were inhibitory, and only zUra was bactericidal. The analogs 6-azathymine and 6-azacytidine were much less inhibitory. Because thymine and probably cytosine were not incorporated, analogs of these bases would be expected to be less inhibitory than analogs of uracil. Analogs such as 2-thiouracil and 6-methyluracil, which contained exocyclic substitutions, were also much less inhibitory than was zUra. Therefore, it appears that purine analogs are more active than the pyrimidine analogs and that, like the purine analogs, pyrimidines with endocyclic substitutions are more inhibitory than analogs with exocyclic substitutions.

Protection by natural bases. The ability of natural bases to protect *M. voltae* from inhibition by zn_2Pur, zGua, zHyp, zUra, or Shy was determined to characterize further the salvage of exogenous bases. Inhibition by the guanine analogs zn_2Pur and zGua was prevented by excess amounts of adenine, guanine, and hypoxanthine (Table 4). Although some eubacteria and at least one methanogen catabolize purines to xanthine (6, 22), the interconversion of free bases for biosynthesis is not common (21). Therefore, the ability of adenine and hypoxanthine to protect the wild type from zn_2Pur and zGua suggests that AMP, IMP, and GMP may be

TABLE 4. Protection of wild-type *M. voltae* by natural bases in the presence of the analogs[a]

Addition to medium	Protection in the presence of the following analogs[b]:				
	zn_2Pur	zGua	zHyp	zUra	Shy
None	−	−	−	−	−
Adenine	+	+	−	−	ND[c]
Guanine	+	+	−	−	−
Hypoxanthine	+	+	+	−	−
Uracil	−	−	−	+	−
Adenine and guanine	ND	ND	−	ND	ND

[a] The analogs were present at concentrations of four times their MICs for the wild type. The natural bases were present at concentrations of 12 times the MIC of the analog being tested.
[b] Symbols: −, no growth after 72 h; +, growth after 24 h.
[c] ND, Not determined.

interconverted in *M. voltae*. This conclusion was also supported by the observation that radiolabeled guanine was incorporated into dAMP in DNA (data not shown). Alternatively, protection could occur if high intracellular concentrations of AMP or IMP inhibited the guanine phosphoribosyltransferase and thus prevented incorporation of zGua. However, experiments in cell extracts will be necessary to distinguish between these possibilities.

In contrast, inhibition by zHyp was prevented only by hypoxanthine, which confirmed that it was a specific analog of this base. The inability of adenine and guanine to protect, either alone or in combination, suggests that they were not converted to IMP. None of the natural bases were protected from inhibition by Shy. The protective capacity of adenine against this analog, however, was not determined because adenine alone was inhibitory at concentrations greater than the MIC for Shy. Inhibition by zUra was prevented only by uracil. Neither cytosine nor thymine protected *M. voltae* from inhibition by zUra (data not shown). This result was consistent with its action as a uracil analog.

Resistant mutants. The ability of the analogs to select for resistant mutants was assayed. Between several hundred and several thousand isolated colonies were obtained on solid medium containing 1 mg of zn_2Pur, zGua, zHyp, zUra, or Shy per ml. One colony was subcultured from each plate. The phenotypes of these mutants were designated Znp^r, Zgu^r, Zhy^r, Zur^r, and Shy^r, respectively. The frequency of these colonies, about 1 in 10^6 to 10^7, suggests that they arose from spontaneous mutants. No colonies were found on plates containing zAde and 2-amino-6-methylmercaptopurine, even though these analogs were only partially inhibitory in liquid culture (Table 2). The MICs for the mutants were at least three orders of magnitude higher than the MICs for the wild type, and in the case of zHyp, the MIC was greater than 30,000-fold higher (Table 3).

In methanogens, resistances to antibiotics or bromoethanesulfonate, a powerful inhibitor of methanogenesis, have been acquired by changes in the permeability of the cell envelope (9, 18, 25, 30). These mutants frequently have an altered morphology, a lower plating efficiency, and only a small increase in resistance. These mutants are very different from the base analog-resistant mutants of *M. voltae*. The base analog-resistant mutants were not obviously pleiotrophic, and their growth rates and morphologies were the same as those of the wild type. Moreover, the mutants acquired very high levels of resistance (Table 3). These results suggest that resistance is probably not due to a change in permeability.

To distinguish between the types of mutants obtained, their cross resistance and ability to incorporate natural bases were determined. Although cross resistance was observed for all the purine analogs, the pattern of resistance differed for some of the mutants (Table 3). The Zgu^r and Zhy^r mutants were resistant to all four purine analogs (Table 3) and zAde (data not shown). In contrast, the Shy^r mutant was only resistant to zn_2Pur, zGua, zHyp, and Shy. The Znp^r mutant was resistant only to zn_2Pur. The Zur^r mutant did not exhibit cross resistance with any of the purines. Therefore, the mutants demonstrated four phenotypes represented by Znp^r, Zur^r, Shy^r, and Zgu^r and Zhy^r, which appeared to be identical.

This pattern of cross resistance allowed for the selection of 11 doubly resistant mutants, all of which were obtained by plating the singly resistant mutants on solid medium containing the inhibitory analogs (Table 3). The MICs determined for the reciprocal double mutants were identical (Table 3), however, which suggests that there is no appreciable difference in their phenotypes.

For many base analogs, the analog is inactive until it is converted into a nucleotide by the salvage pathway. This conversion is referred to as lethal synthesis (20). Inhibition then occurs because of action of the nucleotide analog itself or on incorporation of the analog into DNA or RNA (12, 13, 17, 27). Most of the analog-resistant mutants of *M. voltae* were defective in the salvage pathway for at least one base (Table 1). For instance, the Zur^r mutant could not incorporate uracil. Similarly, three of the mutants of *M. voltae* that were resistant to the purine analogs lost the ability to incorporate some of the purines. Presumably, these mutations also prevented lethal synthesis.

The pattern of incorporation of the natural bases by the resistant mutants supported the conclusions obtained from the cross resistance experiments. The Zur^r mutant was unable to incorporate only uracil. The Znp^r mutant was unable to incorporate guanine (Table 1). However, the incorporation of adenine was unimpaired. This result confirms that zn_2Pur acts as a guanine analog. The Zgu^r and Zhy^r mutants were unable to incorporate guanine and hypoxanthine. Again, the incorporation of adenine was unaffected. To explain the pattern of cross resistance, zAde must also have acted as a guanine or hypoxanthine analog.

In contrast, the incorporation of natural bases was not affected in the Shy^r mutant. Thus, resistance was probably not due to an inability to incorporate the analog. Most of the analogs were insoluble at a concentration of 1 mg/ml. Of special interest, Shy was finely suspended in the agar, which made the medium appear cloudly. When the Shy^r mutant was grown on this cloudly medium, there was a clear zone surrounding the colonies. Therefore, the mutant appeared to remove the analog from the medium. Formation of a clear zone was not observed for the Zgu^r and Zhy^r mutants, which were also cross resistant to Shy. These results further distinguished Znp^r, Zur^r, and Shy^r as distinct phenotypes. *M. vannielii* degrades purine and pyrimidine bases to simple carbon and nitrogen sources (7). However, neither the Shy^r mutant nor wild-type *M. voltae* was able to use Shy or purine bases as sole nitrogen sources (data not shown). Therefore, degradation of Shy may not have been complete. *Escherichia coli* acquires resistance to Shy by converting the thioinosine 5′-monophosphate formed by lethal synthesis to inosine 5′-monophosphate, an important intermediate in purine biosynthesis (4). The activity for this conversion is increased 15-fold in Shy-resistant cells. Because resistance to Shy is lost if *E. coli* is not maintained in the presence of the analog, resistance is acquired by an adaptation to Shy

rather than a specific mutation. Resistance in *M. voltae* was not an adaption, however, because resistance was maintained after repeated transfers in the absence of the analog.

The resistant mutants isolated in this study represent the majority of mutants of *M. voltae* isolated to date. The increase in resistance of these mutants over the wild type is considerably greater than has been observed for those previously isolated resistance mutants in *M. voltae*. The greatly increased resistance, the stability of the mutations, and their unaltered growth characteristics suggest that these analogs may prove to be useful selective agents in the study of methanogen genetics.

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