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5-Fluorouracil-Resistant Strain of *Methanobacterium thermoautotrophicum*

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Growth of *Methanobacterium thermoautotrophicum* Marburg is inhibited by the pyrimidine, 5-fluorouracil (FU). It was shown previously that methanogenesis is not inhibited to the same extent as growth. A spontaneously occurring FU-resistant strain (RTAE-1) was isolated from a culture of strain Marburg. The growth of both strains was inhibited by 5-fluorodeoxyuridine but not 5-fluorocytosine, and the wild type was more susceptible to inhibition by 5-azauracil and 6-azauracil than was strain RTAE-1. The cellular targets for the pyrimidine analogs are not known. When the accumulation of ^{14}C-labeled uracil or FU by the two strains was compared, the wild type took up 15-fold more radiolabel per cell than did the FU-resistant strain. In the wild type, radiolabel from uracil was incorporated into the soluble pool, RNA, and DNA. The metabolism of uracil appeared to involve a uracil phosphoribosyltransferase activity. Strain Marburg extracts contained this enzyme, whereas FU-resistant strain RTAE-1 extracts had less than 1/10 as much activity. Although it is possible that a change in permeability to the compounds plays a role in the stable resistance of strain RTAE-1, the fact that it lacks the ability to metabolize pyrimidines to nucleotides is sufficient to account for its phenotype.

The description of methane-producing archaebacteria is a collection of biochemical variations on familiar themes. Many of the themes have to do with the pathways of methanogenesis and autotrophy and with the cofactors and structural molecules used by the cell (1, 15, 16, 33). Some of the other aspects of metabolism, for example, many of the anabolic reactions and their regulation, repair functions, and salvage mechanisms remain to be clarified. As part of an ongoing study of the coenzymes of methanogens, we chose to work with *Methanobacterium thermoautotrophicum* Marburg, which is an autotroph but appears to take up a number of substrates (7, 8). Enzyme preparations from this thermophile tend to be stable in vitro. Some mutants have been obtained (17). For further genetic work, the plasmid pME2001 and vectors based upon it may be very useful (21, 22).

We previously determined that 5-fluorouracil (FU), a uracil analog, is a potent inhibitor of the growth of *M. thermoautotrophicum* (31). Methanogenesis is not inhibited to the same extent as growth. Here we describe the characterization of a spontaneous isolate which was capable of growth in high concentrations of FU. The characterization of this isolate and its parent provided evidence of the existence of a pyrimidine salvage pathway in methanogenic archaebacteria.

(A preliminary report of some of the results has been presented [D. P. Nagle, Jr., A. Eisenbraun, and R. Teal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I50, p. 173].)

MATERIALS AND METHODS

Sources of chemicals. Bulk chemicals were reagent grade. Uracil, uridine, UMP, FU, 5-fluorodeoxyuridine, and 5-fluorocytosine were from Sigma Chemical Co., St. Louis, Mo.; Omnifluor was from New England Nuclear Corp., Boston, Mass.; [2-^{14}C]FU (58 mCi/mmol) and [2-^{14}C]uracil (53.2 mCi/mmol) were from ICN Radiochemicals, Irvine, Calif.; and phosphoribosylpyrophosphate ($PRPP_i$) and cysteine were from Calbiochem-Behring, San Diego, Calif. Alkaline phosphatase from calf intestine (EC 3.1.3.1) was from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Media and growth conditions. *Escherichia coli* CSH36 (*lacI*) (23) from the department collection (K. Kealy, curator) was grown at 37°C in medium containing, per liter, 5.6 g of KH_2PO_4, 28.9 g of K_2HPO_4, 10 g of yeast extract, 10 mg of thiamine, and 10 g of glucose. *M. thermoautotrophicum* Marburg (DSM2133; obtained from H. Hippe) was grown at 63°C in medium 2 (1) with trace vitamins omitted as described previously (31). Anoxic, sterile solutions of drugs or isotopic tracers were added to 5- or 20-ml cultures. Growth was quantitated by absorbance and converted to number of cells by using a standard curve (31). Large amounts of methanogen cell mass needed for enzyme studies were obtained by using a 3-liter Microferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) maintained at 60 to 63°C and sparged with 1 liter of an 80% H_2–20% CO_2 gas mixture per min.

Isolation of FU-resistant strain. A culture of *M. thermoautotrophicum* Marburg containing 100 μg of FU per ml grew after a prolonged lag period. This culture was continually passaged numerous times in the medium described above with 100 μg of FU per ml. The culture was diluted and plated on medium 2 with 10-fold less Na_2S, 1.5% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.), and 1 mg of FU per ml. The plates were incubated under 200 kPa of an H_2-CO_2-H_2S (80:19:1) gas phase for 2 days at 63°C in stainless steel pressure vessels modified from those described previously (1). A single, well-isolated colony was picked and designated strain RTAE-1.

Uptake of ^{14}C-labeled compounds. For uptake of ^{14}C-labeled compounds, a modification of a previous method was used (2). Membrane filters (pore size 0.45 μm; diameter, 25 mm; Millipore Corp., Bedford, Mass.) were presoaked in buffer (medium 2 without resazurin or cysteine-sulfide reducing solution) containing 1 mg of uracil per ml. At the times indicated, duplicate 0.2-ml samples were anoxically removed from cultures and rapidly vacuum filtered. After

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two 1.0-ml washes with the buffer described above without uracil, the radioactivity trapped on the filters was determined. Each filter was placed in 2.7 ml of Omnifluor scintillation fluid (2.07 g of Omnifluor, 369 ml of Triton X-100, 631 ml of toluene) and 0.3 ml of water in a vial, and the radioactivity was counted in a Beckman LS-100C counter (Beckman Instruments, Inc., Fullerton, Calif.). Values were corrected for radiolabel that bound to filters in the absence of cells. Corrected values for uptake by strain RTAE-1 were at least three times this background, and values for the wild-type strain were correspondingly higher. Quench correction was not required, as determined by the channels-ratio method. In experiments with [2-^{14}C]FU, the radiolabeled compound was added to growing cultures to a final concentration of 41 μM and 1 μCi/ml. In experiments with [2-^{14}C]uracil, the labeled compound was at a final concentration of 48 μM and 0.7 μCi/ml. In the uracil labeling studies, the entire culture was needed for filtration of samples; therefore, growth was monitored by diluting parallel cultures for absorbance measurements. These cultures were prepared and handled identically to those with label except that [^{14}C]uracil (but not [^{12}C]uracil) was omitted.

For determination of the fate of radiolabeled uracil, cultures (20 ml) with 4.1 μM [^{14}C]uracil at 0.22 μCi/ml were grown as described above, centrifuged (10,000 × *g* for 10 min at 4°C), suspended in medium 2 without cysteine-sulfide reducing solution, and recentrifuged. The cell pellet was then fractionated by using a modification of published procedures (32). The cell pellet (40 mg [wet weight]) was suspended in 1 ml of 5% trichloroacetic acid (TCA) and incubated on ice for 30 min. The suspension was then centrifuged (15,600 × *g* for 5 min at 4°C). The supernatant was designated the cold TCA-soluble fraction, and the pellet was resuspended in 1 ml of 5% TCA and incubated in a boiling water bath for 30 min. After centrifugation (as above), the supernatant was designated the hot TCA-soluble fraction. The pellet was suspended in 1 ml of 0.3 M NaOH and designated the TCA-insoluble fraction. The radioactivity in these fractions was determined in triplicate as described above.

Assay of uracil phosphoribosyltransferase. Cells of *E. coli*, *M. thermoautotrophicum* Marburg, and *M. thermoautotrophicum* RTAE-1 were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed by suspension in 100 mM potassium phosphate (pH 7.2) followed by recentrifugation. Cell pellets were stored at −20°C. To prepare extracts, cells were suspended in buffer (50 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, and 7 mM 2-mercaptoethanol for *E. coli*; 50 mM Tris hydrochloride [pH 7.6], 1 mM EDTA, and 5 mM 2-mercaptoethanol for the methanogens). The cells were broken by passage through a French pressure cell two times at 138 MPa. After centrifugation (31,000 × *g* for 30 min at 4°C), crude extracts were stored at −20°C. Protein concentrations were determined by the Coomassie blue dye binding method with bovine serum albumin as the standard (4). Assay mixtures for determining uracil phosphoribosyltransferase activity (100 or 200 μl) contained 50 mM Tris hydrochloride [pH 7.6], 5 mM $MgCl_2$, 200 μM [^{14}C]uracil (1 μCi/ml), 1 mM $PRPP_i$, and 3 to 5 mg of protein per ml. The incubation temperatures were 37°C for *E. coli* and 55°C for *M. thermoautotrophicum* (occasionally 63°C was used for the latter organism, with little difference in results). In some experiments, additional 10-μl samples of $PRPP_i$ (final concentration, 1 mM) were added after 1 h of incubation. Assay mixtures were spotted on cellulose thin-layer chromatography plates (6064; Eastman Kodak Co., Rochester, N.Y.). To detect the nucleotide or nucleoside products, the plates were developed in one of three solvent systems: I, saturated $(NH_4)_2SO_4$-1 M sodium acetate-isopropanol (80:18:2) (10); II, butanol-water (86:14) (10); III, isobutyrate-0.5 N NH_4OH-*n*-butanol (60:36:4) (modified from solvent 3 of Grippo et al. [10]). Known standards were detected visually by UV quenching. The R_f values in these systems, respectively, were as follows: uracil, 0.57, 0.43, and 0.63; uridine, 0.68, 0.30, and 0.63; UMP, 0.72, 0.0, and 0.36. [^{14}C]uracil was shown to be greater than 97% radiochemically pure in system I. Samples of assay mixtures were cochromatographed with 20 nmol of uracil and UMP. The lanes were cut into small segments which were placed in scintillation vials. The radioactivity in the vials was determined by scintillation counting as described above. The counting efficiency (91.1%) was not affected by the presence of the thin-layer sheet or coating. Some samples were treated with alkaline phosphatase by incubation of reaction mixtures with a large excess of alkaline phosphatase in a sealed capillary tube at 37°C. The mixtures were then chromatographed in system III and analyzed as described above.

TABLE 1. Effects of pyrimidine derivatives on growth of FU-resistant *M. thermoautotrophicum* RTAE-1

Addition[a]	Growth (mean [SE])[b]	% of control
None	0.61 (.08)	100
FU	0.53 (.06)	87
5-Fluorodeoxyuridine	0.06 (.02)	10
5-Fluorocytosine	0.57 (.08)	95

[a] Compounds were added at 100 μg/ml. Tubes were incubated for 49 h at 63°C.

[b] Increase in A_{660}. Each value is the mean of at least three individual cultures (± standard error of the mean).

RESULTS

Strain RTAE-1 spontaneously arose from an FU-treated culture of *M. thermoautotrophicum* Marburg and was isolated from a single colony. The strain grew thermophilically, autotrophically, produced methane, and was morphologically identical to strain Marburg. These properties are consistent with strain RTAE-1 being a strain of *M. thermoautotrophicum*. Resistance to FU was maintained after 20 passages in the absence of the compound. The resistant strain grew in the presence of 1 mg of FU per ml, more than 1,000 times the concentration which inhibited the wild type (50% inhibition at 1 μg/ml) (31). When cultures of the wild-type strain were plated on solid medium with 100 μg of FU per ml, the spontaneous mutation rate to FU resistance was determined to be $<1.6 \times 10^{-8}$. Both the wild-type strain and RTAE-1 grew in the presence of 5-fluorocytosine, but neither grew in the presence of 5-fluorodeoxyuridine (Table 1; 31). We also tested 5-azauracil and 6-azauracil as possible inhibitors of both strains. In a 24-h incubation with 600 μg of 5-aza- or 6-azauracil per ml, the yield of wild-type cells was 12 and 22% of that of control cultures (without the compound) respectively, and the yield of strain RTAE-1 was 82 and 76% of that of the control cultures. Thus, the wild type was more susceptible to inhibition by these compounds than was strain RTAE-1 (data not shown).

To determine differences between the wild-type and FU-resistant strains which might be responsible for their phenotypes, growing cultures were incubated with ^{14}C-labeled FU.

In the experiment for which results are shown in Fig. 1, the wild-type strain accumulated significantly more FU than did strain RTAE-1. Growth of the two cultures during the incubation was similar, although it was not quantitated. Based upon the initial cell densities present, at 4 h the wild-type culture had taken up 1.2 nmol/10^8 cells and strain RTAE-1 had taken up 0.07 nmol/10^8 cells, a difference of 17-fold, which was not accounted for by differences in growth.

To avoid the problem of toxicity of FU to the wild type, ^{14}C-labeled uracil was incubated with cells during growth. In the presence of uracil both strains grew at the same rate but clearly took up different amounts of radiolabel (Fig. 2). At 4 h the wild type had taken up 1.3 nmol/10^8 cells (the same amount per cell as in the FU experiment). The ratio of the amount of label in the wild-type cells to that in strain RTAE-1 was more than 5, a value which increased to 15 during the interval from 4 to 12 h. The uptake experiments were initiated by the addition of labeled compound to the cultures. Initial samples were taken as rapidly as possible, but the operations of flushing the syringe with anoxic gas, sampling, and filtration required 2 to 3 min. In this amount of time, cells of both strains bound significant amounts of label, which may have been a combination of adsorption to cells and rapid cellular import. If all the label was transported, the amount taken up by strain RTAE-1 at time zero (0.25 nmol/10^8 cells) corresponded to an internal uracil concentration of 3.6 mM, a value 75 times the external concentration. This calculation was based on assumptions that the labeled compound was not metabolized and that an individual cell weighed 10^{-12} g and consisted of 70% water by weight.

The metabolic fate of labeled uracil in strain Marburg was determined. Labeled cell pellets which had taken up 89% of the uracil in the medium were subjected to TCA fractionation. We found that 29.7% of the radioactivity was in the cold TCA-soluble pool, 68.7% was in the hot TCA-soluble pool (mainly RNA), and 1.7% was in the hot TCA-insoluble pool (mainly DNA). These results showed that uracil was taken up and incorporated into macromolecules in *M. thermoautotrophicum*.

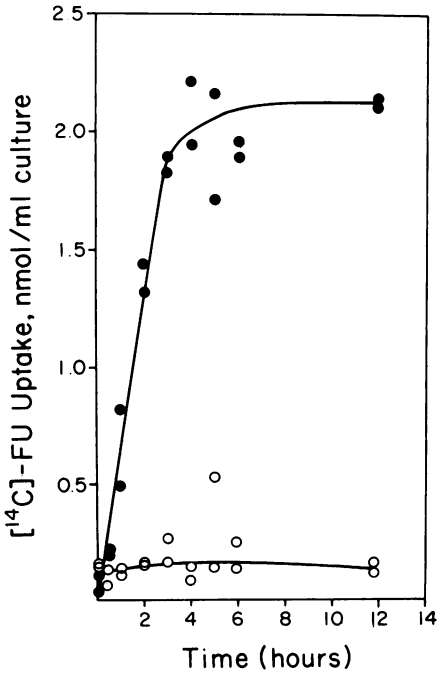

FIG. 1. Uptake of [^{14}C]FU by *M. thermoautotrophicum* Marburg and RTAE-1. Growing cultures were treated with 41 μM [^{14}C]FU, 0.2-ml portions were filtered, and the amount of label retained was quantitated. The initial culture densities were 1.7 × 10^8 cells per ml for strain Marburg and 2.2 × 10^8 cells per ml for strain RTAE-1. Symbols: ●, strain Marburg; ○, strain RTAE-1.

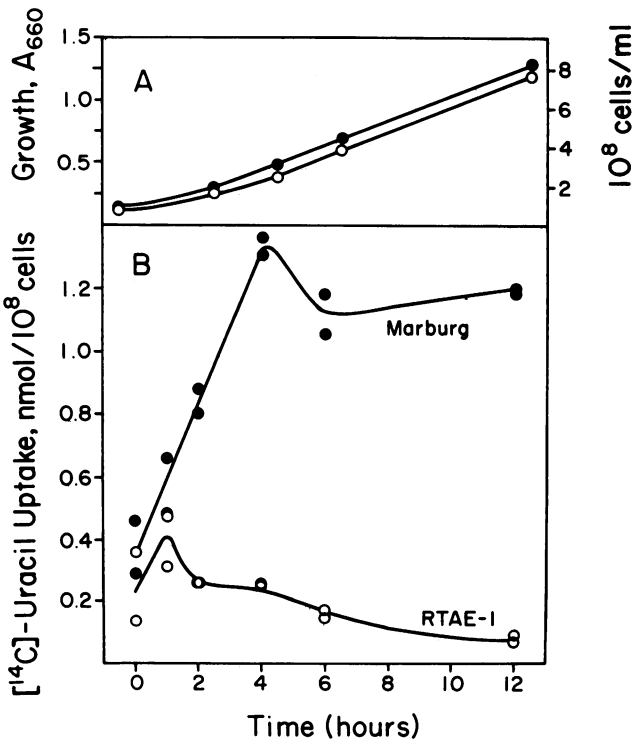

FIG. 2. Uptake of [^{14}C]uracil by *M. thermoautotrophicum* Marburg and RTAE-1. (A) Growth. Cultures with [^{12}C]uracil (identical to those in panel B except that [^{14}C]uracil was omitted) were diluted for absorbance measurements. (B) Label incorporated by cultures treated with [^{14}C]uracil (48 μM). Label was added at time zero, and samples (0.2 ml) from cultures (20 ml) were removed anoxically, filtered, and quantitated. Symbols: ●, strain Marburg; ○, strain RTAE-1.

Since the FU-resistant strain RTAE-1 did not accumulate as much uracil or FU as did the wild type, a defect in metabolism may have been present. Therefore, we tested extracts for the presence of an enzymatic activity which could salvage uracil. Extracts of the wild-type strain contained significant levels of $PRPP_i$-dependent, UMP-forming activity (15% conversion of UMP in 1 h) (Table 2). Extracts of strain RTAE-1 had less than 10% of the activity of the wild type. When extracts of the two strains were mixed, there was no significant decrease in the activity of the wild-type strain, thus ruling out the presence of an inhibitor of the activity in the cell extracts of strain RTAE-1. The product of the reaction, UMP, was identified by comigration of radioactive material from reactions of wild-type extracts with authentic material in solvent systems I, II, and III. There was no significant conversion of UMP to other compounds under the assay conditions. Further evidence that UMP was the product was provided by alkaline phosphatase treatment of the products in reaction mixtures. Radioactivity comigrating with UMP was completely (>99%) converted to a compound which comigrated with uridine after alkaline phosphatase treatment. When ribose-1-phosphate was sub-

TABLE 2. Uracil phosphoribosyltransferase activity in extracts of wild-type and FU-resistant *M. thermoautotrophicum* strains

Organism and assay condition[a]	Incubation time (h)	nmol of UMP/mg of protein
M. thermoautotrophicum Marburg		
Complete	1.0	6.3
$-PRPP_i$	1.0	0.1
Complete	6.0	12.6
$-PRPP_i$	6.0	2.0
$-PRPP_i$, +ribose-1-phosphate	6.0	0.5
Complete + RTAE-1 extract	6.0	11.5[b]
M. thermoautotrophicum RTAE-1	1.0	0.5
(complete)	6.0	1.2
E. coli (complete)	1.0	1.1
	6.0	1.8

[a] Complete assay mixtures included [^{14}C]uracil, $PRPP_i$, $MgCl_2$, buffer, and protein (3 to 5 mg/ml) in 100 μl. The methanogen extracts were incubated at 55°C, and the *E. coli* extracts were incubated at 37°C. After incubation, portions were spotted on cellulose thin-layer plates and chromatographed in solvent system I, and the radioactivity in segments of the lane was quantitated. The amount of UMP synthesized was corrected for that which appeared in incubations without enzyme.

[b] Calculated on the basis of strain Marburg protein present; an equal portion of strain RTAE-1 extract was added.

stituted for $PRPP_i$ in the extract from strain Marburg, there was no evidence for formation of the nucleoside uridine.

The enzyme activity in the wild type was oxygen stable but decreased upon repeated thawing and freezing of extracts. The results shown in Table 2 were from a typical experiment. The amount of UMP synthesized in 1 h by wild-type extracts varied from 5 to 10 nmol/mg, but strain RTAE-1 extracts never formed more than 0.5 nmol/mg. There was no significant difference in activity in incubations done at 55 and 63°C and no variability which could be ascribed to differences in extracts from independently grown cells. However, the activities were not computed as rates because the amount of UMP synthesized was not linearly proportional to time or to the amount of protein. The labile nature of the substrate $PRPP_i$ probably contributed to this observation. At the elevated temperatures required for the thermophilic organism, $PRPP_i$ appeared to be limiting. In some experiments in which additional $PRPP_i$ was added to assay mixtures at 15-min intervals, we obtained UMP synthesis levels 2 to 5 times higher than those shown in Table 2 and as much as 63% substrate conversion in 3 h. *E. coli*, used as a control organism, had low levels of enzyme activity (Table 2). We did not attempt to optimize assay or growth conditions with *M. thermoautotrophicum* or *E. coli*.

DISCUSSION

FU inhibits the growth of *M. thermoautotrophicum*. We have previously shown that this drug does not act directly on methanogenesis (31). Since uracil but not cytosine, thymine, uridine, or thymidine spared the cells from inhibition, there is specificity in transport or metabolism of FU (31). Since labeled uracil was taken up and incorporated into RNA and DNA, the organism must have a mechanism to salvage uracil. The data in Table 2 show that part of the uracil activation system in the wild type was a $PRPP_i$-dependent, UMP-forming enzyme activity. The amounts of activity detected were significantly less than those in a eubacterium which requires preformed pyrimidines (20), in *E. coli* or *Salmonella typhimurium* strains induced for the enzyme (3, 5, 27), or in the yeast *Saccharomyces cerevisiae* (24). However, they were the same as those found in cultured plant cells which do not require pyrimidines (14). *M. thermoautotrophicum* is an autotroph and might not be expected to need much pyrimidine salvage activity.

The mechanisms by which FU and the azauracil compounds inhibit the growth of *M. thermoautotrophicum* are not known. In eubacteria and eucaryotes, nucleotide metabolites of these compounds are the actual inhibitors. For example, FU is metabolized to 5-fluorodeoxyuridylate, a potent inhibitor of thymidylate synthesis. Without thymidylate, DNA synthesis cannot occur (12). FU is also metabolized to 5-fluorouridine triphosphate and is incorporated into RNA, and FU-substituted RNAs cannot function as efficiently as normal forms (19). We have observed that thymine or thymidine do not spare the cells from inhibition by FU (31). If either of these is activated to the nucleotide form, DNA synthesis ought to be able to continue despite the presence of FU. Thus, it is possible that FU metabolites incorporated into RNA disrupt its function in the methanogen. The azauracil compounds are activated to nucleotide derivatives which inhibit de novo pyrimidine biosynthesis (28). We have not studied the reversal of inhibition by the azauracil compounds.

The FU-resistant strain RTAE-1 grew at the normal rate at levels of FU 10^3 times those which inhibited growth of the wild type. The strain was also less susceptible to inhibition of growth by 5-azauracil and 6-azauracil than was the wild-type strain. When strain RTAE-1 was grown in the presence of radiolabeled uracil or FU, the cells failed to accumulate label. The amounts incorporated at the first sampling time appeared to be more than could be accounted for by nonspecific binding, which ought to increase as the number of cells increases. These data might be explained by altered permeability to the compounds in strain RTAE-1. Resistance to pyrimidine analogs in certain mutants of the fungi *Neurospora crassa* and *S. cerevisiae* is accounted for by decreased permeability (6, 9). However, since extracts of strain RTAE-1 contained less than 10% as much uracil phosphoribosyltransferase activity as did the wild type, it appeared that the uracil analogs were not activated. Both FU resistance in the eubacteria (Upp phenotype) and 6-azauracil resistance in plant tissue culture cells result from the loss of uracil phosphoribosyltransferase (3, 5, 14, 25, 26). Definitive proof that the resistance of the strain was not due to a combination of lowered permeability and loss of uracil phosphoribosyltransferase activity will require transport studies. However, the decrease in the levels of the uracil salvage enzyme in the FU-resistant strain RTAE-1 may be sufficient to account for its resistant phenotype.

Mutation to drug resistance may result from changes in the genes for (i) transport of the compound, (ii) the site of action, or (iii) activation of the compound to the inhibitory form. Metabolic inactivation by a degradative enzyme activity will not be considered here. For methanogens, alternative i holds for mutants with resistance to neomycin or virginiamycin (*Methanococcus voltae*) or bromoethanesulfate (*M. voltae* and *Methanosarcina barkeri*). In these instances, the evidence suggests that the resistant cell is less permeable to the compound than is the wild type (11, 29, 30). A similar explanation has been proposed for other resistances (11, 17).

There are two examples of mechanism ii, altered site of action, in methanogens. The ribosome of an anisomycin-resistant *Methanobacterium formicicum* strain is not inhibited by the compound; the cells are cross-resistant to several

other ribosome inhibitors (13). The isoleucyl-tRNA synthetase in a pseudomonic acid-resistant *M. thermoautotrophicum* strain is not inhibited by the compound, unlike the wild-type enzyme (18).

FU-resistant strain RTAE-1 appears to be an example of alternative iii, resistance to an antimetabolite because of a deficient activation process. Strain RTAE-1 was deficient in uracil phosphoribosyltransferase and refractory to inhibition by FU and azauracils. It is likely that the type of pyrimidine (and probably purine) analog resistance, "namely, failure to form fraudulent ribonucleotide derivatives from the corresponding base" (5), found in strain RTAE-1 will be common in archaebacteria as it is in eubacteria and eucaryotes. Purine and pyrimidine analog resistances have been reported for strains of *M. voltae* (T. L. Bowen and W. B. Whitman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986. I47, p. 172), and we have isolated 6-mercaptopurine- and 8-azaguanine-resistant *M. thermoautotrophicum* strains (V. Worrell, D. McCarthy, and D. P. Nagle, Jr., unpublished results). Work is in progress to define the site of action of FU and to develop a genetic exchange system based on our resistant strains.

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