JOURNAL OF BACTERIOLOGY, Dec. 1989, p. 6534–6538
0021-9193/89/126534-05$02.00/0

Vol. 171, No. 12

Formate Auxotroph of *Methanobacterium thermoautotrophicum* Marburg

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Received 15 May 1989/Accepted 6 September 1989

A formate-requiring auxotroph of *Methanobacterium thermoautotrophicum* Marburg was isolated after hydroxylamine mutagenesis and bacitracin selection. The requirement for formate is unique and specific; combined pools of other volatile fatty acids, amino acids, vitamins, and nitrogen bases did not substitute for formate. Compared with those of the wild type, cell extracts of the formate auxotroph were deficient in formate dehydrogenase activity, but cells of all of the strains examined catalyzed a formate-carbon dioxide exchange activity. All of the strains examined took up a small amount (200 to 260 μmol/liter) of formate (3 mM) added to medium. The results of the study of this novel auxotroph indicate a role for formate in biosynthetic reactions in this methanogen. Moreover, because methanogenesis from H_2-CO_2 is not impaired in the mutant, free formate is not an intermediate in the reduction of CO_2 to CH_4.

The autotrophic, thermophilic archaebacterium *Methanobacterium thermoautotrophicum* has several attractive features for the development of a genetic system in a methanogen, including well-characterized mutants (19, 21), the presence of a cryptic plasmid (17) which may be suitable for the development of a plasmid vector (18), and a primitive natural transformation system for genetic exchange (30). While much of the research into developing a genetic exchange system in *M. thermoautotrophicum* in our laboratory has focused on mutants resistant to nitrogen base analogs (19, 28, 30; V. E. Worrell and D. P. Nagle, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, I22, p. 221; D. P. Nagle, Jr., Dev. Ind. Microbiol., in press), mutants with auxotrophic markers would also be useful for these investigations into the genetics of *M. thermoautotrophicum*. In this report we describe the isolation and partial characterization of a unique auxotroph, a formate-requiring strain of *M. thermoautotrophicum*.

(A portion of this work has been presented elsewhere [R. S. Tanner and D. P. Nagle, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, I10, p. 182].)

MATERIALS AND METHODS

Strains and media. Strains of *M. thermoautotrophicum* used in this study were ΔH (ATCC 29096), obtained from R. S. Wolfe; Marburg (DSM 2133), obtained from H. Hippe; K^r, a kanamycin-resistant derivative of Marburg; and RT-103, a formate-requiring derivative of K^r. *M. thermoautotrophicum* K^r was isolated after prolonged incubation of a culture in medium containing 500 μg of kanamycin per ml. The MICs of kanamycin sulfate in basal medium for strains Marburg and K^r were 175 and 1,000 μg/ml, respectively.

The basal medium contained the following, in grams per liter: NaCl, 0.8; NH_4Cl, 1.0; KCl, 0.1; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $NaHCO_3$, 5.0; resazurin, 0.0005; cysteine hydrochloride, 0.4; and $Na_2S \cdot 9H_2O$, 0.4, as well as 10 ml of trace metal solution. The trace metal solution was adapted from that of Wolin et al. (29) and contained the following, in grams per liter: nitrilotriacetic acid, 2.0 (pH adjusted to 6 with KOH); $MnSO_4 \cdot H_2O$, 1.0; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.8; $CoCl_2 \cdot 6H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.2; $CuCl_2 \cdot 2H_2O$, 0.02; $NiCl_2 \cdot 6H_2O$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.02; Na_2SeO_4, 0.02; and Na_2WO_4, 0.02. For the growth of kanamycin-resistant strains, anoxic filter-sterilized kanamycin sulfate (Boehringer Mannheim Biochemicals) was added to the medium to a final concentration of 500 μg/ml after the medium was autoclaved. The ability of strains of *M. thermoautotrophicum* to use formate (74 mM) as a sole source of carbon or reducing equivalents was tested in a bicarbonate-free medium incubated with one of the following gas phases: H_2, N_2, or N_2-CO_2 (80:20). In this medium, $NaHCO_3$ was replaced with 44 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; pH 7.3).

Vitamin, amino acid, nitrogen base, and volatile fatty acid (VFA) solutions were added to basal medium for auxotroph selection and characterization. The vitamin solution was adapted from that of Wolin et al. (29) and was added to give the following final concentration of each component, in micrograms per liter: pyridoxine hydrochloride, 100; 2-mercaptoethanesulfonic acid, 100; vitamin B_{12}, 50; thiamine hydrochloride, 50; riboflavin, 50; calcium pantothenate, 50; *p*-aminobenzoic acid, 50; thioctic acid, 50; nicotinic acid, 50; biotin, 20; and folic acid, 20. The amino acid solution was added to give the following final concentration of each component, in grams per liter: vitamin assay Casamino Acids (Difco Laboratories) (20), 4.0; tryptophan, 0.01; methionine, 0.02; asparagine, 0.1; and glutamine, 0.1. The nitrogen base solution was added to give final concentrations of 50 mg each of uracil, cytosine, thymine, adenine, and guanine per liter. The VFA solution was adapted from those previously described (1, 14) and contained formate, which had previously been added to VFA solutions for culture of methanogens from the ruminal environment (M. P. Bryant, personal communication) and was added in this study to give the following final concentrations of each component, in grams per liter: sodium formate, 2.5; sodium acetate, 2.5; propionic acid, 1.0; butyric acid, 0.6; isobutyric acid, 0.2; valeric acid, 0.2; isovaleric acid, 0.2; and 2-methylbutyric acid, 0.2.

For plate medium, 20 g of purified agar (Difco) per liter was added, the levels of cysteine and sodium sulfide were reduced to 0.04 g/liter, and the plates were incubated with an atmosphere of H_2-CO_2-H_2S (79:20:1) (1, 2). Media and

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cultures were prepared and handled by strict anoxic techniques (1, 2). Cultures were incubated at 60°C with an H_2-CO_2 (80:20) atmosphere pressurized to 300 kPa (2).

Mutagenesis and auxotroph selection. *M. thermoautotrophicum* K^r cells (10^9) were mutagenized by incubation in 3 M $NH_2OH \cdot HCl$ (4 ml), which had been adjusted to pH 6.0, for 20 min at 37°C. The treated cells were washed and transferred to basal medium (10 ml) containing kanamycin and bacitracin (40 μg/ml) (5, 21). After incubation for 12 h at 60°C, the mutagenized culture was transferred to medium containing kanamycin and each of the nutrient solutions: vitamins, amino acids, nitrogen bases, and VFA. After 24 h of incubation, during which growth occurred, the bacitracin selection procedure was repeated, including a second outgrowth of the culture in nutrient-supplemented medium. Samples of the culture were then plated onto basal agar medium containing kanamycin and all of the nutrient solutions. After growth, colonies were patched onto both the nutrient-containing agar and basal agar medium. Colonies which grew only on the nutrient-supplemented medium were transferred to liquid medium containing all of the nutrient solutions and kanamycin.

Auxotroph characterization. The nature of the auxotrophic requirement of selected colonies was determined by elimination of a nutrient pool from nutrient-supplemented medium followed by examination of the components of the nutrient solution required. Sodium pyruvate (P 2256; Sigma Chemical Co.) was also tested (up to 45 mM).

Labeling experiments. To examine the role of formate in the metabolism of *M. thermoautotrophicum*, [^{14}C]formate (CFA.11; Amersham Corp.) was added to 20-ml cultures in 160-ml serum bottles. Approximately 7% of the radioactivity in the [^{14}C]formate was present as [^{14}C]HCO_3^-; this was determined by ion exclusion high-pressure liquid chromatography with a radiochemical detector (P. S. Beaty, personal communication). Samples were taken at different times, and the radioactivity in the gas, supernatant, and cell pellet was determined; cells were pelleted by centrifugation at 16,000 × *g* for 2 min at room temperature. Radioactivity was monitored with a liquid scintillation counter (LS 1701; Beckman Instruments, Inc.) with a toluene–Triton X-100 (2:1) solvent mix containing 3 g of Omnifluor (Du Pont, NEN Research Products) per liter as a scintillant. Total radioactivity in the gas phase was estimated as described by Zehnder et al. (31). Radioactivity in CO_2 was then determined by absorption into a 0.2 N NaOH solution (for a 0.5-ml gas sample, 100 μl of base was used) which was then subjected to liquid scintillation counting. Radioactivity in methane was calculated as the difference between the radioactivity in the gas sample and that absorbed into the base. Cells of strains of *M. thermoautotrophicum* grown in the presence of [^{14}C]formate were fractionated as described by Hespell et al. (9, 10) to determine the distribution of radioactivity in various cell components. Methane was measured by gas chromatography. Formate was measured by ion exclusion high-pressure liquid chromatography on an Aminex HPX-87C column (Bio-Rad Laboratories) monitored at A_{214} with 0.01 N H_2SO_4 as the eluant.

FDH activity. Strains of *M. thermoautotrophicum* were mass cultured by using a 10-liter Biostat E (B. Braun). Cells were harvested by continuous centrifugation and suspended in anoxic 20 mM TES buffer, pH 7.0 (1 ml/g [wet weight] of cells). Cells were lysed by anoxic passage through a French pressure cell (110,000 kPa), and cell extract (CFE) was obtained after anoxic centrifugation at 48,000 × *g* at 4°C for 30 min. The CFE was stored at −20°C under N_2. Protein in the CFE was estimated by measuring the turbidity at 400 nm of a sample in 20% trichloroacetic acid. Buffers used for formate dehydrogenase (FDH) assay were 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.5), TES (pH 7.5), and *N*-tris(hyroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS; pH 8.5). The pHs of stock buffers were adjusted at room temperature. To measure FDH activity, each anoxic assay was prepared under N_2 in sealed cuvettes and contained 2.9 μmol of TES, 0.57 μmol of dithiothreitol, 57 μmol of assay buffer, 1.1 μmol of $MgCl_2 \cdot 6H_2O$, 6.0 μmol of electron acceptor, 1 mg of protein (from the CFE), and 57 μmol of sodium formate in a final volume of 2.6 ml. FDH activity was measured at 60°C with a spectrophotometer (DU 64; Beckman) equipped with a water-jacketed cuvette holder. Each assay was preincubated at 60°C without CFE and formate. CFE was added, and a background rate of reduction of the electron acceptor was measured for 6 min. Formate was added, and the reaction rate was monitored for another 6 min. Specific activity was calculated as the net rate of reduction of the electron acceptor (in nanomoles per minute per milligram of protein) in the presence of formate and was corrected for the background reduction rate. The reduction of NAD and NADP was monitored at 340 nm. Buffers and electron acceptors were obtained from Sigma.

RESULTS AND DISCUSSION

Isolation of auxotrophs. *M. thermoautotrophicum* K^r was mutagenized with hydroxylamine, and auxotrophs were enriched for using bacitracin as described in Materials and Methods. After outgrowth in basal medium containing all of the nutrient solutions, approximately 8,000 cells from each trial were plated onto agar medium of the same composition. For the strains described here (all from a single trial), 96 of the 200 colonies that appeared on plates were examined for an auxotrophic requirement. Of these 96 colonies, 24 were auxotrophs and were found to require formate for growth. One strain, RT-103, was selected for further characterization since it was readily cultured on basal medium supplemented with only kanamycin and sodium formate (15 to 37 mM).

Formate requirement. Growth and methanogenesis from H_2-CO_2 in formate-supplemented medium by strain RT-103 were essentially identical to growth and methanogenesis observed for the parent strain, K^r. Under the conditions used for the labeling experiments, the times required for an A_{660} increase of 0.1 (1.6-cm light path) in basal medium with 15 mM formate were 1.07 and 0.97 h for strains K^r and RT-103, respectively; the increases in absorbance after 46 h of incubation were 2.3 and 2.4, respectively. When strain K^r was grown in the basal medium without formate, the time required for an absorbance increase of 0.1 was 0.98 h, and the absorbance after 46 h of incubation was 2.5. The rate of methanogenesis averaged from 200 to 400 μmol/h in these cultures. Growth and methanogenesis were not observed in cultures of strain RT-103 if formate was omitted from the medium.

The requirement for formate was absolute; RT-103 did not grow on medium containing the vitamin solution, amino acid solution, nitrogen base solution, pyruvate, and all of the other VFAs except formate. The concentration of formate required for growth of strain RT-103 was >0.15 mM. Good growth occurred within 24 h in tubes containing 2.9 to 15 mM formate. Growth was delayed until 72 h in cultures containing 0.74 or 1.5 mM formate; once growth occurred, the rate and amount of growth were the same as in cultures containing 15 mM formate. The formate auxotrophy was maintained

TABLE 1. Metabolism of [^{14}C]formate by *M. thermoautotrophicum*

Strain[a]	Radiolabel in fraction[b] (10^6 dpm)				
	Initial (supernatant)	Final			
		Supernatant[c]	Pellet[d]	CO_2	CH_4
ΔH	45	2.4	1.6	2.3	24
Marburg	38	1.5	1.5	3.2	22
K^r	62	2.1	2.4	6.5	47
RT-103	62	2.2	2.2	3.3	42

[a] Strains of *M. thermoautotrophicum* are described in Materials and Methods.
[b] [^{14}C]formate was added to 10-ml cultures containing 3.7 mM formate to an approximate specific activity of 1.5×10^6 dpm/μmol. Cultures were pressurized to 220 kPa overpressure with H_2-CO_2 (80:20) at 0, 16, and 40 h.
[c] Final number was determined after 46 h of incubation.
[d] Initial radioactivity in the cell pellets was $<0.02 \times 10^6$ dpm.

by plating and picking colonies on agar medium with and without formate every 6 months. Three-tube most-probable-number determinations (3) indicated a reversion frequency of 2×10^{-7} for loss of the auxotrophic requirement.

[^{14}C]formate-labeling studies. The metabolism of formate in strains of *M. thermoautotrophicum* was examined by using radiolabeled substrate. It had been reported that *M. thermoautotrophicum* could incorporate label from [^{14}C]formate into cellular components, particularly purines and histidine (27). Under the experimental conditions used here, label from [^{14}C]formate (initial concentration of 3.7 mM, with a specific radioactivity of 1.0×10^5 dpm/μmol) was incorporated into the following cellular components by strains K^r and RT-103, respectively (in 10^4 dpm/10^{10} cells): soluble pool, 3.8 and 7.4; lipid, 6.9 and 6.8; RNA, 1.9 and 2.8; DNA, 0.9 and 1.0; protein, 2.2 and 1.7. These data show that radioactivity from formate was incorporated into all the cellular components examined and that the pattern of labeling was similar for both strain K^r and the formate auxotroph.

The fate of label from [^{14}C]formate in strains of *M. thermoautotrophicum* is shown in Table 1. Only about 3.6 to 3.9% of the original radioactivity added was apparently taken up by the cells. Most of the radioactivity was recovered in CH_4 at the end of incubation; this was an unexpected result, since *M. thermoautotrophicum* does not use formate for methanogenesis (32). However, the metabolism of [^{14}C]formate was similar in the strains of *M. thermoautotrophicum* examined, and the data suggested the exchange of radiolabel between formate and CO_2.

A time course for the biotransformation of radioactivity from [^{14}C]formate is shown in Table 2. The results suggest that the radioactivity in formate was in partial equilibrium with the CO_2-HCO_3^- pool. This would explain the radioactivity subsequently found in CH_4 or incorporated into cellular material.

TABLE 2. Metabolism of [^{14}C]formate by *M. thermoautotrophicum* K^r

Time (h)	Radiolabel in fraction[a] (10^6 dpm)			
	Supernatant	Pellet	CO_2	CH_4
0	9.6	0.00019	0.68	<0.002
20	8.7	0.0036	0.75	0.31
28	7.4	0.024	1.6	1.2
44	1.1	0.44	0.96	6.7
60	0.44	0.57	0.091	8.1

[a] Initial concentration of [^{14}C]formate (1.7×10^5 dpm/μmol) was 2.9 mM in 20-ml cultures maintained at a constant overpressure of 220 kPa with H_2-CO_2.

TABLE 3. FDH activity in *M. thermoautotrophicum* CFE

Strain[a]	Buffer pH[b]	Electron acceptor(s)	Sp act[c]
Marburg	8.5	NAD, NADP	0.8
	7.5	NAD, NADP	2.4
	6.5	NAD, NADP	6.6
	6.5	NAD	7.8
	6.5	NADP	8.4
RT-103	6.5	NAD	−0.6
	6.5	NADP	2.8

[a] Strains are described in Materials and Methods. Cells were lysed by anoxic passage through a French pressure cell, and CFE was obtained after anoxic centrifugation at 48,000 × *g* at 4°C for 30 min.
[b] Stock buffer (TAPS, TES, or MES) at room temperature.
[c] Assay details are given in Materials and Methods. Each assay, complete except for the addition of formate, was incubated at 60°C to determine the background rate of reduction of the electron acceptor(s) added at a final concentration of 20 mM. The specific activity is the net rate of reduction of the electron acceptor(s) (in nanomoles per minute per milligram of protein) in the presence of formate, corrected for the background rate.

This exchange reaction was unexpected. To substantiate that the conversion of radioactivity from formate to CO_2-HCO_3^- was due to an exchange reaction and not to the direct metabolism of formate, the amount of formate in supernatant fractions was measured by high-pressure liquid chromatography. When the initial concentration of formate was 2.9 mM, the decreases in the concentration of formate in 48-h cultures of strains ΔH, Marburg, K^r, and RT-103 were 0.25, 0.20, 0.22, and 0.26 mM, respectively. It had been previously reported that *M. thermoautotrophicum* does not assimilate significant amounts of formate (26). In our study, under conditions in which >95% of the radioactivity disappeared from the supernatant, <10% of the formate was removed from the supernatant. The ability of strains of *M. thermoautotrophicum* to use formate as a sole source of carbon or reducing equivalents for growth or methanogenesis was also examined, as described in Materials and Methods. There was no evidence for growth or methane production from formate in the four strains examined (data not shown), which agrees with earlier work (32). This exchange reaction will require further characterization. Studies with [^{14}C]formate to determine its direct role in the metabolism of *M. thermoautotrophicum* will probably require assay conditions uncomplicated by the presence of a formate-CO_2 exchange reaction. However, the experiments described above showed that formate was metabolized in a similar fashion by each of the four strains of *M. thermoautotrophicum* examined.

FDH activity. The formate required by strain RT-103 could be produced by the direct reduction of CO_2 in the wild type. Assays were performed in the reverse direction to determine whether this occurred as FDH activity. FDH activity was detected in the CFE of *M. thermoautotrophicum* Marburg (Table 3). This activity was influenced by assay pH, being most active at the lowest pH examined. The observed activity was dependent on the presence of NAD or NADP as an electron acceptor and was directly proportional to the amount of CFE added to the assay (data not shown). No activity was detected when flavin adenine dinucleotide, flavin mononucleotide, or benzyl viologen was used as an electron acceptor (phenazine methosulfate and dichlorophenolindophenol were unstable under the assay conditions).

NAD-dependent FDH activity was not detected in the

CFE of strain RT-103. This strain had lower levels of NADP-dependent FDH activity than Marburg did. These observations are consistent with the hypothesis that a mutation in the formate auxotroph affected the activity of an FDH system, resulting in a need for formate in the medium to support growth. When methyl viologen was used as the electron acceptor (W. H. Lorowitz, D. P. Nagle, Jr., and R. S. Tanner, unpublished results), mean FDH activities (with 95% confidence limits) were 3.2 ± 1.7 and 0.86 ± 0.25 nmol/min per mg of protein in CFEs of strains Marburg and RT-103, respectively. The low level of activity is consistent with a biosynthetic rather than a catabolic role for FDH in *M. thermoautotrophicum* when compared with the level found in a formate-utilizing species such as *Methanobacterium formicicum* (830 nmol/min per mg of crude extract protein) (23). Elucidation of the exact nature of FDH in *M. thermoautotrophicum* will require further work, including purification of the activity and examination of FDH in a revertant of RT-103. The detection of FDH activity and a formate-CO_2 exchange reaction in *M. thermoautotrophicum* might be due to the inclusion of tungsten and selenium in addition to molybdenum (common to many trace metal solutions) in the medium used for culture of these strains. These metals may be required for FDH activity (16).

This is the first report of a formate auxotroph in a bacterial species. The requirement for formate in *M. thermoautotrophicum* RT-103 is specific. Without formate, the combination of pyruvate and all of the components of the nutrient solutions (vitamins, amino acids, nitrogen bases, and VFA) did not support growth of this strain. An auxotrophic requirement for formate in *M. thermoautotrophicum* was unexpected. This microorganism uses H_2-CO_2, not formate, for methanogenesis and growth (32; also see above). Formate is not a free intermediate in methanogenesis (4, 22, 24), but there are two formyl carriers, methanofuran (15) and tetrahydromethanopterin (6), in the pathway for the reduction of CO_2 to CH_4 in this microorganism (22). Since methanogenesis from H_2-CO_2 is apparently not affected in *M. thermoautotrophicum* RT-103, the formyl groups formed during methanogenesis probably cannot be used for certain biosynthetic reactions.

A role for formate in biosynthesis in *M. thermoautotrophicum* is not known. The primary CO_2-fixing pathway in *M. thermoautotrophicum*, the pathway for the biosynthesis of acetyl coenzyme A, is well documented (8, 11, 25, 26). Formate does not appear to be a free intermediate for incorporation into either carbon of the acetyl group (11, 25, 26). Determination of a biosynthetic role for formate in *M. thermoautotrophicum* by substitution with other compounds may be difficult, since this microorganism probably lacks appropriate uptake mechanisms (7, 12), growth can be inhibited by relatively high (millimolar) concentrations of organic compounds in media (12, 13), and more than one compound may be required.

This novel formate auxotroph will be important for further research into the genetics and molecular biology of this thermophilic, methanogenic archaebacterium (17, 18, 30; Nagle, in press) whose physiology and biochemistry have been studied extensively (8, 22), and for further studies in one-carbon metabolism, based on the utilization of formate, in methanogens.

ACKNOWLEDGMENTS

This research was supported by grant N00014-86-K-0222 from the Office of Naval Research. Additional support was provided by grant DMB-8404907 from the National Science Foundation.

We thank Shi Lui for technical assistance with a radiolabel experiment and Bill Lorowitz and Dave McCarthy for helpful discussions.

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