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Methanosarcina Mutant Unable to Produce Methane or Assimilate Carbon from Acetate

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Mutants of *Methanosarcina barkeri* 227 resistant to monofluoroacetate were isolated from monofluoroacetate-treated cultures. Mutant strain FA^{r}9 was 100 times more resistant to monofluoroacetate than the wild-type strain and was deficient in carbon uptake and CH_4 and CO_2 production from methyl-labeled acetate. Methanol was assimilated at increased levels. Strain FA^{r}9 was unable to shift from using methanol to using acetate for growth and exhibited increased sensitivity to growth inhibition by NaCN in methanol-containing complex medium. Unlike parent strain 227, acetate addition to methanol-containing media did not prevent NaCN inhibition. The specific activities of enzymes of exogenous acetate assimilation, CO dehydrogenase, and enzymes of the tricarboxylic acid cycle were similar for mutant and parent strain cell extracts. Mutation to monofluoroacetate resistance did not confer simultaneous resistance to 2-bromoethanesulfonate or pyruvate or alter propionate uptake. We conclude that strain FA^{r}9 is either an acetate permeability mutant or is defective in an activation step required for the catabolism and anabolism of acetate.

Acetate is a key intermediate in methanogenic metabolism (1, 25). Methanogens use acetate as a carbon source and may require it as a growth factor (1, 25). Those methanogens that do not require acetate for growth synthesize it (or acetyl coenzyme A [CoA]) from one-carbon substrates (12). Acetyl-CoA and pyruvate are key points on the pathway of acetate carbon metabolism and provide the carbon needed for the synthesis of cellular components and intermediates (12, 15, 28, 37). The tricarboxylic acid (TCA) cycle of methanogenic bacteria is incomplete and is used primarily for biosynthesis (1).

Methanosarcina barkeri produces CH_4 from H_2-CO_2, methanol, methylamines, or acetate and synthesizes cell carbon from methanol plus CO_2 or from CO_2 alone (18). The synthesis of cell carbon from one-carbon precursors is thought to involve the action of a carbon monoxide dehydrogenase-containing enzyme complex analogous to that of *Methanobacterium thermoautotrophicum* and acetogenic bacteria (16, 29). The product of the CO dehydrogenase enzyme complex has not been determined for *Methanosarcina barkeri*, but it is acetyl-CoA in *Methanobacterium thermoautotrophicum* (12). Acetyl-CoA is assimilated after reductive carboxylation to pyruvate and via formation of TCA cycle intermediates (12, 15, 25, 37).

Methanosarcina barkeri may also assimilate exogenous sources of acetate into cell carbon (20, 33). Cell-free extracts contain high specific activities of acetate kinase and phosphotransacetylase, which together may allow the synthesis of acetyl-CoA from acetate (18). Acetyl-CoA synthetase activity has not been reported in *Methanosarcina barkeri* although the activity of this enzyme is implicated in exogenous acetate assimilation by *Methanobacterium thermoautotrophicum* (18, 28). A few methanogens (*Methanosarcina* spp. and *Methanothrix soehngenii*) use acetate as an energy substrate and produce methane from it (13, 24, 26, 32, 35). Methane is produced from acetate by a decarboxylation reaction (aceticlastic reaction) in which the methyl group is reduced to CH_4 and the carboxyl group is oxidized to CO_2 (26). The free energy yield is too low ($\Delta G'^{o} = -36$ kJ) to permit the synthesis by substrate level phosphorylation of 1 mol of ATP per mol of acetate cleaved, and a chemiosmotic mechanism of ATP synthesis has been postulated (7, 36). It is assumed that methanogenic cell membranes are freely permeable to acetate because the low energy yield of the aceticlastic reaction precludes active transport of acetate into cells for cells growing on acetate as an energy source (38).

The biochemical steps of carbon metabolism in methanogens are not completely understood, and the steps in methanogenesis from acetate are mainly speculative (1). In vitro systems in which methane is produced from acetate have been described recently (2, 22). In many cases novel pathways and coenzymes are involved in metabolism, and it is not known whether exchange occurs between catabolic pathways and biosynthetic pathways via common intermediates (1, 18). Mutants in transport and biochemical pathways are needed to provide markers for future genetic studies and to work out the biochemical pathways in metabolism. Unfortunately, the genetics of methanogens is in its infancy, and few mutants are available (3, 19, 34; G. Bertani and L. Baresi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I73, p. 133).

In this paper we describe the first reported isolation of a mutant methanogenic bacterium which is not able to catabolize a methanogenic substrate. We show that the mutation has multiple effects on acetate utilization and that the properties of the mutant are consistent with a loss of permeability of *Methanosarcina barkeri* to acetate or with a defect in an activation step needed for methanogenesis and carbon assimilation from acetate.

(Part of this work has been reported previously [M. R. Smith and J. L. Lequerica, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, I12, p. 148].)

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MATERIALS AND METHODS

Bacterial strains and culture media. *Methanosarcina barkeri* 227 (= DMS 1538) was isolated from an acetate enrichment culture (26).

Cultures were maintained by transferring them at intervals of 3 days in MOPS [3-(*N*-morpholino)propanesulfonic acid] complex basal medium (31) containing 40 mM methanol. MOPS complex basal medium (complex medium) contained 1.0 g of NH_4Cl per liter, 0.4 g of K_2HPO_4 per liter, 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter, 2.62 g of MOPS buffer per liter, 2.0 g of yeast extract per liter, 2.0 g of Trypticase per liter, 0.3 g of NaOH per liter, 0.5 g of cysteine hydrochloride per liter, 0.0001 g of resazurin per liter, and 1,000 ml of water. The final pH before autoclaving was 7.1 to 7.2. MOPS complex basal medium was used for routine transfers and for all experiments in which complex medium was used.

For some experiments, cultures were grown in defined medium containing 1.0 g of NH_4Cl per liter, 0.4 g of K_2HPO_4 per liter, 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter, 2.62 g of MOPS per liter, 0.3 g of NaOH per liter, 0.5 g of cysteine hydrochloride per liter, 0.0001 g of resazurin per liter, and 1,000 ml of water; the pH was 7.1 to 7.2. Defined medium was supplemented with (per 100 ml of medium) 10 ml of Wolin mineral solution and 10 ml of Wolin vitamin solution (1) before boiling under N_2 to eliminate oxygen.

Cysteine hydrochloride was added to complex or defined medium after boiling. Reagent grade chemicals and distilled water were used for all preparations. The media were dispensed anaerobically into serum bottles, which were stoppered with butyl rubber stoppers, and autoclaved for 15 min at 121°C. Growth substrates (methanol or acetate) and a sodium sulfide-bicarbonate solution (10% [wt/vol] $NaHCO_3$, 1% [wt/vol] $Na_2S \cdot 9H_2O$) were added from sterile anaerobic stock solutions just before inoculation to give 100-fold-lower concentrations in the final medium.

Growth. Growth was monitored by measuring methane production and the increase in absorbance at 450 nm. Specific growth rate constants (μ; ln 2/doubling time) were calculated from the exponential increases in methane or absorbance levels during incubation of the cultures. The specific growth rate constants obtained by the two methods were in good agreement.

Experiments to determine the effect of fluoroacetate (FA) on growth were performed by inoculating cultures into complex medium containing 40 mM methanol plus 2 mM acetate (added as a component of Trypticase [33]), 40 mM methanol plus 40 mM sodium acetate, or 40 mM sodium acetate alone and 0, 10, 100, or 1,000 μM sodium monofluoroacetate. Cultures containing methanol were inoculated with methanol-grown inocula, and cultures containing acetate were inoculated with acetate-grown inocula. Cultures were incubated at 35°C, and methane production was monitored daily for 3 to 14 days depending on whether the cultures contained methanol or acetate.

The effects of inhibitors on growth were examined in cultures (50-ml portions in 160-ml serum bottles) to which concentrated, filter-sterilized, anaerobic stock solutions of inhibitors in distilled water had been added to the desired concentrations. After inoculation (1% volume) the cultures were incubated at 35°C for 3 to 5 days, and levels of methane production and absorbance were monitored at intervals during incubation.

Chemicals. The chemicals and enzymes used for enzyme activity assays, sodium 2-bromoethanesulfonate (BES), and sodium FA were purchased from Sigma Chemical Co., St. Louis, Mo. 2,5-Diphenyloxazole (PPO) and 1,4-bis-[2]-(5-phenyloxazolyl)benzene (POPOP) were purchased from Packard Instrument Co., Inc., Downers Grove, Ill. The protein dye-binding reagent used for protein determinations was purchased from Bio-Rad Laboratories, Richmond, Calif.

Isotopes. [^{14}C]methanol (58 mCi/mmol), sodium [2-^{14}C]propionate (44 mCi/mmol), and sodium [2-^{14}C]acetate (57.5 mCi/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. Solutions were prepared and filter sterilized anaerobically in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.).

Cell-free extracts. Cell-free extracts were prepared anaerobically from cell suspensions grown in 10 liters of MOPS complex medium containing 40 mM methanol (30). All procedures were carried out in a Coy model A anaerobic glove box containing an H_2-N_2 atmosphere. Cell pellets (8 to 10 g, wet weight) were washed once in PD buffer (20 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$, 2 mM dithiothreitol), 20 mM tricine–KOH buffer (pH 7.2), or distilled water. The pellets were used immediately for extract preparation, or sterile glycerol was added to a final concentration of 10%, followed by storage in liquid N_2.

Extracts were prepared by suspending the pellets in PD buffer, tricine-KOH buffer, or distilled water, followed by disruption under a stream of H_2 in a French press at 135 mPa. Cell breakage was estimated by phase-contrast microscopy. The broken cells were centrifuged anaerobically at 30,000 × *g* for 30 min at 4°C. The supernatant was collected and used for enzyme assays immediately or kept under liquid N_2 until it was used. Enzyme activities were stable for at least 2 weeks in liquid N_2.

Phosphate-free extracts were prepared by the method of Eyzaguirre et al. (11) by passing exracts anaerobically through a column of Dowex AG1-X8 (20/50 mesh; acetate form; Bio-Rad).

Gases. Gases (CH_4 and CO_2) were determined by constant volume sampling of culture headspaces and gas chromatography as described previously (32). $^{14}CH_4$ and $^{14}CO_2$ were determined with a Packard model 894 gas proportional counter connected in tandem to a thermoconductivity gas chromatograph as described previously (32).

Labeling experiments. Radioactive labeling studies were performed as described previously (33) to determine the fate of [^{14}C]methanol, [2-^{14}C]acetate, and [2-^{14}C]propionate in cultures of strains 227 and FA^{r}9 growing on a mixture of methanol and acetate (or propionate). These experiments were performed in duplicate by using 50-ml portions of complex medium in 160-ml serum bottles. Experiments were repeated at least once, with good agreement between experimental runs. Methanol (40 mM) plus acetate (10 mM) or propionate (2 mM) was added as the energy substrate. The media also received 5 μCi of [^{14}C]methanol, sodium [2-^{14}C]acetate, or sodium [2-^{14}C]propionate before inoculation. A 1% (vol/vol) inoculum of a methanol culture of strain 227 or FA^{r}9 was used for inoculation, and the cultures were incubated at 35°C for 162 h. Methane production, $^{14}CH_4$ production, and $^{14}CO_2$ production were monitored at 24-h intervals during the incubation period; culture samples (1 ml) were removed at the end of incubation to determine the quantity of acetate assimilated into cell carbon. The samples were filtered onto 0.45-μm polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) and washed once with 10 ml of 5% trichloroacetic acid and three times with distilled water (10 ml each). The filters were dried for 3 days in a desiccator and then counted with a Packard Tricarb model

460 liquid scintillation counter by using Aquasol (New England Nuclear Corp., Boston, Mass.) as the counting fluid. The remaining culture was acidified by adding 1 ml of 20 N H_2SO_4. The acidified cultures were shaken at intervals for 2 h before determining final $^{14}CH_4$ and $^{14}CO_2$ levels; $^{14}CH_4$ and $^{14}CO_2$ levels were again determined at 24 h after acidification. There were no significant differences between the two determinations.

The effect of acetate concentration on the quantity of acetate assimilated was determined in 25-ml methanol cultures in 50-ml serum bottles. Cultures grown on 40 mM methanol in defined medium were used to inoculate (1%, vol/vol) 25 ml of defined medium containing 20 mM methanol and 1 to 40 mM sodium acetate. Anaerobic, filter-sterilized sodium [2-^{14}C]acetate (5 μCi) was added to each serum bottle before inoculation. The cultures were then incubated at 35°C for 72 to 96 h, and the methane level was monitored at intervals. Cells were harvested by filtration at the end of the incubation period, acidified with 5% trichloroacetic acid, washed, dried, and counted by liquid scintillation counting. Methane production was exponential until incubation was terminated.

For the assimilation studies final cell masses were estimated from the final quantity of methane produced during incubation. Separate experiments were performed without labeled substrate to determine the quantity of cells (in grams) produced per mole of CH_4 (growth efficiency) for each acetate concentration used in the experiments. Growth efficiency was found to be constant under the conditions of the experiments. The amount of acetate (in micromoles) assimilated per gram of cells was then calculated from the amount of radioactivity taken up and the quantity of methane produced.

Assays. Protein levels in cell-free extracts were measured by the dye-binding procedure of Bradford (5).

The levels of acetate in culture fluids were determined by gas chromatography (33) with a detection limit of 3 nmol (1 μM concentration in the original culture fluid).

Enzyme assays were performed anaerobically at 37°C by using N_2 as the gas phase and 1-cm quartz cuvettes sealed with soft rubber stoppers. Changes in absorbance were monitored with a recording spectrophotometer. Reagents for enzyme assays were prepared in an anaerobic glove box and injected into the cuvettes with a syringe. The final reaction volumes were 1 ml. Assays were conducted over the range where activity varied linearly with protein concentration.

Acetate kinase activity was measured in the direction of acetate formation and in the direction of acetyl phosphate formation. Acetate kinase assays in the direction of acetate synthesis depended on the coupling of acetate kinase activity to the reduction of NADP (365 nm; ε = 3.4 cm^2/μmol) via hexokinase and glucose-6-phosphate dehydrogenase. Ap_5A (Sigma) was added as an inhibitor of myokinase, which interferes with the assay. The reaction mixture contained 100 mM tricine–KOH buffer (pH 7.5), 2 mM ADP, 2 mM glutathione, 5 mM $MgCl_2$, 1 mM NADP, 0.2 mM Ap_5A, 2.8 U of hexokinase, 1.4 U of glucose-6-phosphate dehydrogenase, 10 mM glucose, 0.1 to 0.4 mg of extract protein, and 2 mM acetyl phosphate. The assay was started by injecting acetyl phosphate into reaction mixtures prewarmed to 37°C. Acetate kinase activity in the direction of acetyl phosphate synthesis was measured by coupling the reaction to the oxidation of NADH (365 nm; ε = 3.4 cm^2/μmol) by pyruvate kinase and lactic dehydrogenase. The reaction mixtures contained 100 mM tricine–KOH buffer (pH 7.5), 5 mM ATP, 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 20 mM sodium acetate, 0.4 mM NADH, 0.7 U of pyruvate kinase, 6 U of lactic dehydrogenase, and 0.1 to 0.4 mg of cell extract protein. The assay was started by injecting acetate after first measuring ATPase activity. ATPase activity was estimated by following the rate of oxidation of NADH in the acetate kinase assay mixture (acetyl phosphate synthesis) before adding acetate.

Acetate thiokinase (EC 6.2.1.1) activity was measured by the procedure of Oberlies et al. (28).

Phosphotransacetylase (EC 2.3.1.8) activity was assayed spectrophotometrically (29) at 233 nm (ε = 4.44 cm^2/μmol) by following the formation of acetyl-CoA from acetyl phosphate and CoA. The reaction mixture contained 100 mM tricine–KOH buffer (pH 7.5), 100 mM $(NH_4)_2SO_4$, 0.2 mM Co-A, 2 mM acetyl phosphate, and cell extract (50 to 200 μg of protein). The assay was started by injecting acetyl phosphate into the reaction mixture.

Adenylate kinase (myokinase) (EC 2.7.4.3) activity was determined by coupling the formation of ATP from ADP to reduction of NADP by hexokinase and glucose-6-phosphate dehydrogenase (29). The reaction mixture consisted of 100 mM tricine–KOH buffer (pH 7.5), 5 mM $MgCl_2$, 2 mM glutathione, 20 mM glucose, 1 mM NADP, 2 mM ADP, 0.7 U of hexokinase, 0.7 U of glucose-6-phosphate dehydrogenase, and cell extract (0.1 to 0.5 mg of protein). Ap_5A (0.2 mM) was also added as a control. The assay was initiated by injecting an ADP solution into the reaction mixture at 37°C and monitoring NADPH formation at 365 nm.

The pyruvate synthase and α-ketoglutarate dehydrogenase assays used were the methyl viologen assays of Zeikus et al. (39). Succinate dehydrogenase (EC 1.3.99.1) activity was determined by following fumarate-dependent oxidation of reduced benzyl viologen (39).

Succinate thiokinase (EC 6.2.1.5) activity was based on the succinate-dependent formation of ADP from ATP and CoA (28). The phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38) assays used depended on the phosphoenolpyruvate-, phosphate-, and CO_2-dependent formation of oxaloacetate (17). Pyruvate phosphate dikinase was assayed by using phosphate-free extracts and following pyruvate-, Mg^{2+}-, ATP-, and phosphate-dependent formation of phosphoenolpyruvate (8). Pyruvate kinase (EC 2.7.1.40) was determined for pyruvate synthesis by following phosphoenolpyruvate-, ADP-, Mg^{2+}-, and K^+-dependent formation of pyruvate (4). In the direction of phosphoenolpyruvate formation, pyruvate kinase was estimated by using the pyruvate phosphate dikinase assay and crude extracts containing phosphate (8).

Carbon monoxide dehydrogenase activity was assayed spectrophotometrically at 578 nm by following CO-dependent reduction of methyl viologen (ε = 9.7 cm^2/μmol) (6). Assays were conducted in 9-ml test tubes closed with rubber stoppers. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.5), 40 mM 2-mercaptoethanol, 1 mM methyl viologen, and 50 to 200 μg of cell extract protein; the final reaction volume was 3 ml. The gas phase over the incubation mixture (nitrogen) was changed to CO by flushing. The tubes were warmed to 37°C, and the contents were shaken. The reaction was started by adding cell extract. Activity was calculated from the following stoichiometry: 2 mol of methyl viologen reduced per mol of CO oxidized to CO_2.

Citrate synthase (EC 4.1.3.7) was determined in a coupled spectrophotometric assay in which oxaloacetate was formed from malate via malate dehydrogenase (8). The reaction

mixture contained 100 mM tricine–KOH buffer (pH 8.2), 20 mM sodium malate, 10 mM NH_4Cl, 0.4 mM NAD, 0.2 mM acetyl-CoA, 40 mM mercaptoethanol, 5.6 U of malate dehydrogenase, and cell extract (1 mg of protein). The reaction was started by injecting acetyl-CoA after incubating without acetyl-CoA for 20 min to obtain a stable base line. NADH formation was monitored at 365 nm.

Malate dehydrogenase (EC 1.1.1.42) was assayed by using isocitrate-dependent reduction of NADP to NADPH at 340 nm ($\varepsilon = 6.22\ cm^2/\mu mol$) (37). The reaction mixture contained 100 mM tricine–KOH buffer (pH 7.5), 5 mM $MnCl_2$, 0.25 mM NADP, 5 mM dithiothreitol, 1 mM threo-D_SL_S-isocitrate, and cell extract (0.1 mg of protein). Assays were started by injecting threo-D_SL_S-isocitrate.

Aconitase (EC 4.2.1.3) activity was determined by coupling citrate-dependent isocitrate formation with the isocitrate dehydrogenase reaction (37). The reaction mixture consisted of 100 mM tricine–KOH buffer (pH 7.5), 5 mM $MnCl_2$, 0.25 mM NADP, 0.44 U of isocitrate dehydrogenase, 2 mM sodium citrate, and cell extract (0.1 to 0.2 mg of protein). The reaction was started by adding sodium citrate.

RESULTS

FA inhibition in *Methanosarcina barkeri* 227 and development of spontaneous mutants. Growth inhibition of strain 227 in complex medium depended on the ratio of acetate to FA. For 40 mM methanol cultures containing 2 mM acetate, the growth rate was inhibited 77% by 100 μM FA. When sodium acetate was added to a final concentration of 40 mM, inhibition of the growth rate decreased to only 8%. In contrast, cultures growing on 40 mM sodium acetate as the sole energy source exhibited 90% inhibition of the growth rate in the presence of FA compared with untreated controls. This indicates that FA adversely affected carbon assimilation and methanogenesis from acetate but was less inhibitory to methanogenesis from methanol.

We observed that methanol cultures inhibited by 0.1 to 1 mM FA grew after 9 days of incubation. The cultures had become resistant to 100 μM FA and grew without a lag in FA-methanol-containing medium after being transferred repeatedly (more than 25 transfers) in the absence of FA. Serial dilution of parent strain 227 cultures into liquid complex medium with and without FA indicated that the fraction of the parent strain population represented by FA-resistant mutants was 10^{-4}.

FA-resistant colonies were isolated from a methanol culture of strain 227 that had developed resistance to 100 μM FA. Isolation was accomplished by inoculating serial 10-fold dilutions of the resistant culture into roll tubes containing methanol but no added FA. Colonies that developed after incubation of the roll tubes were picked from the tube containing the highest dilution exhibiting growth (10^5) and were inoculated into liquid medium containing 40 mM methanol but no FA. One such FA-resistant mutant was designated strain FA^r9. FA resistance was retained after 25 transfers in liquid methanol-containing medium without FA. Strain FA^r9 cultures were maintained by routine transfer in methanol-containing medium without FA.

We found no differences between mutant and parent culture growth rates when H_2-CO_2, methanol, and trimethylamine were used as energy sources. The growth rates, growth efficiency (in moles of CH_4 per gram of cells), and optimal pH for growth (pH 6.4) were constant and the same for parent and mutant cultures growing on methanol in complex or defined medium.

Effects of FA on parent and mutant cultures. In MOPS complex medium containing 40 mM methanol, the kinetics of methanogenesis by strain 227 were exponential at FA concentrations below 100 μM (Table 1). At concentrations of 100 μM or greater linear kinetics of methanogenesis were observed; the rate decreased approximately twofold for each 10-fold increase in FA concentration. Linear methane production did not occur in strain FA^r9 until FA concentrations reached 10 mM or more. The change from exponential to linear methane production by strain 227 suggests that FA completely inhibited growth but not methanogenesis, and visible growth was not observed during linear methanogenesis. Decreasing rates of methanogenesis with increasing FA concentrations indicated that some inhibition of methanogenesis occurred at high FA concentrations.

TABLE 1. Effects of inhibitors in MOPS complex basal medium on the growth of *Methanosarcina*[a]

Addition	Concn (mM)	μ (h^{-1})[b]	
		Strain 227	Strain FA^r9
Sodium FA	0	0.056	0.065
	0.1	L[c]	0.065
	1	L	0.062
	10	L	L
Sodium pyruvate	0	0.066	0.067
	1	0.060	0.063
	5	0.039	0.051
	10	0.039	0.047
	25	0.036	0.041
	50	0.022	0.030

[a] MOPS complex basal medium contained 40 mM methanol and 2 mM acetate.
[b] Calculated from the exponential increase in methane levels.
[c] L, Linear methane production.

Strain FA^r9 was similar to strain 227 in its sensitivity to sodium pyruvate (Table 1). Pyruvate (5 mM) inhibited growth of the parent and mutant strains approximately 40% compared with untreated controls; little inhibition occurred at a pyruvate concentration of 1 mM. This indicates that no changes occurred in the sensitivity of strain 227 to pyruvate as a result of the mutation to FA resistance. It is notable that the level of sensitivity of strains 227 and FA^r9 to inhibition by pyruvate is approximately 1 order of magnitude greater than the levels reported for other methanogens (14). The increase in growth inhibition caused by pyruvate at concentrations above 20 mM was probably caused by Na^+ because NaCl controls exhibited inhibitory effects on growth of the parent and mutant strains at concentrations above 25 mM.

Strain FA^r9 did not show increased resistance to BES compared with the parent strain, and BES-resistant permeability mutants (30) exhibited parent strain sensitivity to inhibition by FA. This indicates that there were separate mutational events for the two types of mutants which coexisted in the parent strain population.

Fate of carbon from acetate in strains 227 and FA^r9. In *Methanosarcina*, acetate can be used catabolically for ATP and methane production or anabolically for cell carbon synthesis. Growth experiments (data not shown) in which methanol cultures of strains 227 and FA^r9 were used to inoculate complex medium containing 20 mM methanol and 20 mM sodium acetate showed that strain 227 could reproducibly shift from using methanol to using acetate as an energy source, while strain FA^r9 could not. The inability of strain FA^r9 to shift to using acetate for growth could be due to defects in enzymes of acetate catabolism, in regulatory

TABLE 2. Partitioning of carbon from methanol, acetate, and propionate into metabolic products in *Methanosarcina*[a]

Strain	Labeled substrate	μ (h^{-1})	Amt of $^{14}CH_4$ (μmol)	Amt of label incorporated (mmol/g of cells)		
				$^{14}CH_4$	$^{14}CO_2$	Cells
227	[^{14}C]methanol	0.063	715	104	24.8	3.5
	[2-^{14}C]acetate	0.061	664	1.9	1.5	8.1
	[2-^{14}C]propionate	0.076	534	ND[b]	ND	0.15
FA^{r}9	[^{14}C]methanol	0.052	619	106	33.6	7.3
	[2-^{14}C]acetate	0.056	659	ND	ND	0.08
	[2-^{14}C]propionate	0.070	474	ND	ND	0.12

[a] The values are averages of duplicate determinations. Complex basal medium was supplmented with 40 mM methanol and 10 mM sodium acetate or 2 mM sodium propionate.
[b] ND, Not detected.

genes of acetate metabolism, or in the permeability of the cells to acetate.

Radioactive labeling experiments with [^{14}C]methanol or sodium [2-^{14}C]acetate in complex medium containing 40 mM methanol and 10 mM acetate as growth substrates were undertaken to determine whether strain FA^{r}9 was capable of assimilating acetate or of producing methane from it.

Table 2 shows that parent strain 227 grew normally and produced $^{14}CH_4$ and $^{14}CO_2$ from [^{14}C]methanol and [2-^{14}C]acetate on the methanol-acetate mixture. The methyl carbon atom of acetate (8.1 μmol of ^{14}C per mg of cells) was preferred over [^{14}C]methanol (3.5 μmol of ^{14}C per mg of cells) as a source of cell carbon. Strain FA^{r}9 exhibited a quite different partitioning of carbon from [^{14}C]methanol and [2-^{14}C]acetate into cells and gases. In strain FA^{r}9, [^{14}C]methanol went to $^{14}CH_4$ and $^{14}CO_2$ as it did in strain 227, except that greater quantities of $^{14}CO_2$ were produced from radioactive methanol. The quantity of [^{14}C]methanol assimilated by strain FA^{r}9 into cell carbon was approximately double the amount assimilated by strain 227. Formation of radioactive methane and CO_2 from methyl-labeled acetate was not detected in our experiments with strain FA^{r}9, and uptake of methyl-labeled acetate into cell carbon by strain FA^{r}9 was reduced 100-fold compared with the parent strain. An analysis of the culture medium by high-pressure liquid chromatography before and after growth of the cultures showed that strain 227 consumed more than one-half of the radioactive acetate added, while strain FA^{r}9 consumed an undetectable amount. This indicates that the mutation to FA resistance affected both catabolic and anabolic pathways of acetate utilization and that acetate was not metabolized by the mutant to a soluble intermediate which accumulated in the culture medium.

The levels of sodium [2-^{14}C]propionate assimilation by the parent and mutant cultures did not differ significantly and were similar in magnitude to the quantities assimilated by *Methanobacterium thermoautotrophicum* (9).

Effect of cyanide on substrate metabolism by strain FA^{r}9. Cyanide (10 μM) has been reported to inhibit methanogenesis from acetate and uptake of methanol into cell carbon in strain 227 (31). At an acetate concentration of 2 mM, acetate assimilation was stimulated by NaCN. These effects were probably the result of NaCN inhibition of carbon monoxide dehydrogenase activity (21). Addition of sodium acetate to cultures prevented NaCN inhibition of growth on methanol in defined medium. In view of these effects of NaCN on the parent *Methanosarcina* strain, we wanted to determine the effects of NaCN on growth and metabolism by the mutant strain.

Table 3 shows the effects of 10 μM NaCN and 10 mM sodium acetate on the growth of strains 227 and FA^{r}9 on methanol in defined medium. NaCN inhibited the growth rates of the parent and mutant cultures 35% compared with untreated controls. Addition of 10 mM sodium acetate to the culture medium abolished NaCN inhibition of growth of the parent strain but did not abolish growth inhibition of the mutant.

In complex medium containing 40 mM methanol, strain FA^{r}9 was more sensitive to growth inhibition by NaCN than strain 227 was. NaCN inhibited the growth rate of strain 227 by only 9%, compared with 49% for strain FA^{r}9.

In radioactive labeling experiments (data not shown), 10 μM NaCN inhibited the assimilation of $^{14}CO_2$ and $^{14}CH_3OH$ into cell carbon by 20% in strain 227 on methanol and acetate in MOPS defined medium but did not inhibit the growth rate or the amount of carbon assimilated from acetate. In contrast, NaCN inhibited the growth rate of strain FA^{r}9; the quantity of carbon per gram (dry weight) of cells assimilated from methanol decreased, but the amount of carbon incorporated from the methyl group of acetate increased fourfold. However, the amount of carbon assimilated from acetate in the presence of NaCN was only 5% of the amount taken up by the parent strain.

Effect of acetate concentration on carbon assimilated from acetate. At acetate concentrations less than 2 mM, uptake of [2-^{14}C]acetate into cell carbon in defined medium increased nonlinearly with acetate concentration for both parent and mutant cultures (Fig. 1A and B). When acetate concentrations were increased over the range from 1 to 40 mM, methyl group uptake was proportional to acetate concentration (Fig. 1C and D). Although the mutant and parent cultures exhibited similar patterns of acetate assimilation, the mutant assimilated 20- to 100-fold less acetate per gram of cells than the parent strain did at the acetate concentrations tested.

The possibility that strain FA^{r}9 overproduced acetate from methanol was tested by comparing the levels of acetate excreted into the culture medium by the parent and mutant strains growing on methanol in defined medium. Acetate was not detected in either culture.

Enzymes of acetate assimilation in strains FA^{r}9 and 227. The multiple effects of the mutation to FA resistance suggest a block in acetate utilization that is common to anabolic and catabolic pathways.

Table 4 shows the levels of enzyme activity in mutant and parent strain extracts. The greatest differences in specific activity were found for isocitrate dehydrogenase and for

TABLE 3. Effect of 10 μM NaCN on growth of strains 227 and FA^{r}9 on 40 mM methanol in defined medium[a]

Addition(s)	Strain 227		Strain FA^{r}9	
	μ (h^{-1})	Final A_{450}	μ (h^{-1})	Final A_{450}
None (untreated control)	0.049	0.156	0.048	0.116
NaCN (10 μM)	0.032	0.033	0.031	0.014
Sodium acetate (10 mM)	0.059	0.274	0.052	0.115
Sodium acetate (10 mM) plus NaCN (10 μM)	0.059	0.352	0.031	0.019

[a] The defined medium contained 40 mM methanol. Incubation was terminated on the same day for all cultures. The differences in the final A_{450} values between strains for control (untreated) cultures reflect differences in inoculum size. The values are averages of duplicate determinations.

enzymes in the pathway for conversion of acetate to acetyl-CoA. The specific activities for acetate kinase, phosphotransacetylase, and adenylate kinase in the wild type did not exceed four times those in the mutant extracts. Mutant and parent extracts had similar activities for pyruvate synthesis from acetyl-CoA (pyruvate synthase) and for two enzyme activities detected for the synthesis of phosphoenolpyruvate from pyruvate (pyruvate kinase and pyruvate phosphate dikinase). Pyruvate phosphate dikinase activity has not been reported previously for extracts of *Methanosarcina*. Enzymes for conversion of pyruvate or acetyl-CoA to TCA intermediates (citrate synthase) and enzymes of the TCA cycle (aconitase and malate dehydrogenase) were at similar levels in mutant and parent extracts. Other enzyme activities, such as acetate thiokinase, phosphoenolpyruvate carboxytransphosphorylase, succinyl dehydrogenase, succinate thiokinase, and α-ketoglutarate dehydrogenase, were not detected in either the parent or mutant cell extracts. CO dehydrogenase activity, which functions in the synthesis of acetate from one-carbon units in acetogenic bacteria and probably also in methanogenic bacteria (16, 29), was present at nearly the same levels in the mutant and parent strain extracts.

DISCUSSION

The FA-resistant mutant described here and the BES permeability mutants described previously (30, 34) were isolated as spontaneous mutants. The relative ease with which *Methanosarcina* BES permeability mutants are isolated (34) suggests that permeability mutations may occur at high rates. Mah et al. (26) suggested that the original culture of *Methanosarcina barkeri* 227 might be genetically heterogeneous because colony counts in roll tube media varied

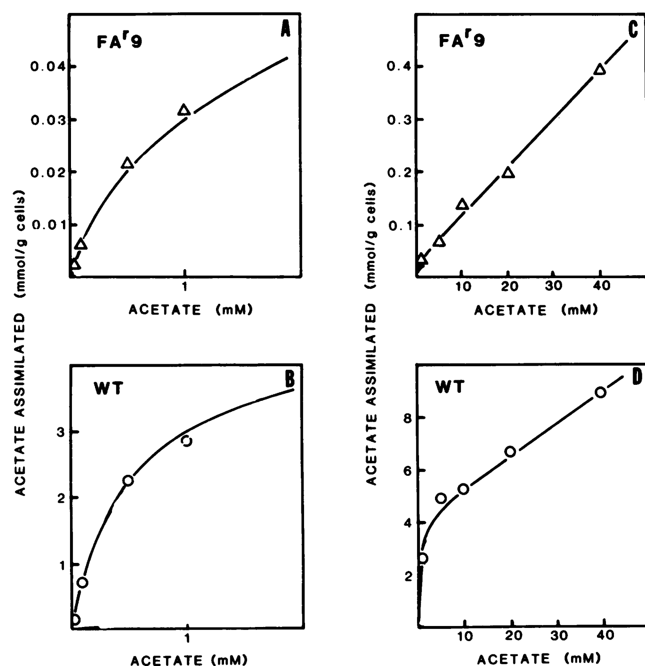

FIG. 1. Effect of acetate concentration on acetate assimilated into cell carbon. (A) Strain FA^{r}9 (0 to 2 mM acetate). (B) Strain 227 (0 to 2 mM acetate). (C) Strain FA^{r}9 (1 to 40 mM acetate). (D) Strain 227 (1 to 40 mM acetate). Symbols: △, strain FA^{r}9; ○, strain 227. Note the different ordinate scales for strains FA^{r}9 and 227. WT, Wild type.

TABLE 4. Enzymatic activities of cell-free extracts of *Methanosarcina barkeri* 227 and FA^{r}9[a]

Enzyme	EC no.	Sp act (nmol/min per mg of protein)		Ratio of strain 227 sp act to strain FA^{r}9 sp act
		Strain 227	Strain FA^{r}9	
Acetate kinase (acetate formation)	2.7.2.1	319	233	1.4
Acetate kinase (acetyl-P formation)	2.7.2.1	85	48	1.8
Phosphotransacetylase	2.3.1.8	3,109	833	3.7
Acetate thiokinase	6.2.1.1	ND[b]	ND	
Adenylate kinase (myokinase)	2.7.4.3	315	191	1.6
ATPase		19	18	1.1
Pyruvate synthase	1.2.—.—	130	175	0.7
Pyruvate phosphate dikinase	2.7.9.1	14	18	0.8
Pyruvate kinase (PEP formation)[c]	2.7.1.40	31	36	0.9
Pyruvate kinase (pyruvate formation)	2.7.1.40	9	9	1.0
PEP carboxytrans-phosphorylase	4.1.1.38	ND	ND	
Citrate synthase	4.1.3.7	2.1	2.6	0.8
Malate dehydrogenase	1.1.1.37	62	50	1.2
Succinyl dehydrogenase	1.3.99.1	ND	ND	
Succinate thiokinase	6.2.1.5	ND	ND	
α-Ketoglutarate dehydrogenase	1.2.—.—	ND	ND	
CO dehydrogenase		689	503	1.4
Aconitase	4.2.1.3	38	38	1.0
NADP-Isocitrate dehydrogenase	1.1.1.42	189	89	2.1

[a] The values represent mean activities of at least three extract preparations. Enzymatic activity in each extract was the average of six determinations.
[b] ND, Activity not detected.
[c] PEP, Phosphoenolpyruvate.

depending on the substrates used for growing and plating inocula. Cultures of *Methanosarcina* grown on H_2-CO_2 or methanol frequently do not grow well on acetate without a long adaptation period (26). It has been suggested that the adaptation period might represent the selection of a small number of mutants in the population that use acetate (20, 26). This implies that most of the *Methanosarcina* populations of methanol cultures are not able to use acetate as a growth substrate. However, our FA-resistant mutants represented only a small fraction of the parent strain population on methanol and could not account for the long period required for methanol cultures to adapt to acetate.

Our results show that strain FA^{r}9 was deficient in utilizing acetate but not one-carbon substrates (H_2-CO_2, methanol, methylamine) for methanogenesis and cell carbon synthesis. The precise nature of the defect is not certain from our data, but our results are consistent with a defect that occurs at an early step in acetate utilization. This defect might be at an early metabolic step necessary for methanogenesis and carbon metabolism or at the level of entry of acetate into cells. Information about acetate uptake and metabolism in *Methanosarcina* is sparse, and strain FA^{r}9 could provide important clues concerning how acetate is utilized.

It is possible that strain FA^{r}9 is defective in acetate metabolism. Since acetate is used both as an energy (e.g., methane) source and as a carbon source, a defect in metab-

olism should occur at a step common to carbon and energy metabolism. Strain FA^{r}9 grew normally on single-carbon substrates, suggesting that pathways for acetate utilization that are shared with single-carbon substrates were not impaired by the mutation to FA resistance.

The terminal step in energy metabolism from CO_2 or methanol is the formation of methyl coenzyme M (1). Recent evidence indicates that methyl coenzyme M formation is also needed for methanogenesis from acetate (23, 30, 32). Methane is produced from acetate by a decarboxylation reaction. The initial cleavage of acetate might require an initial activation step (40). According to current hypotheses of acetate cleavage, an intermediate derived from the acetate methyl group donates its methyl group to coenzyme M for reduction to methane, and a carbonylated intermediate derived from the carboxyl group is oxidized to CO_2 (7, 40). Electrons provided by the oxidation step are used to reduce methyl coenzyme M to methane. A carbon monoxide dehydrogenase-containing enzyme complex is believed to be involved in oxidation of the acetate carboxyl group to CO_2 (10, 27).

Activation of acetate is also needed for assimilating exogenous acetate into cell carbon (18, 37, 40). Initial activation is believed to be by formation of acetyl-CoA via acetate kinase and phosphotransacetylase reactions (18, 37). These two enzymes are present at high specific activities in *Methanosarcina* cell extracts, particularly when the extracts are from acetate-grown cultures (18, 37). Acetyl-CoA is assimilated into cell carbon via the TCA cycle and by reductive carboxylation to pyruvate (12, 15, 37).

Evidence suggests that *Methanosarcina* carbon metabolism from single-carbon substrates also begins with the formation of acetate or acetyl-CoA (18). In *Methanobacterium thermoautotrophicum* acetyl-CoA is the initial product of CO_2 fixation by carbon monoxide dehydrogenase-containing enzyme complexes analogous to those of acetogenic bacteria (12, 29). If methanol is converted to two-carbon units by formation of acetyl-CoA, then the phosphotransacetylase and acetate kinase steps of exogenous acetate metabolism are involved uniquely in uptake of exogenous acetate. The other biosynthetic steps are thought to be shared for acetate and one-carbon substrates (18, 37).

In the discussion above we assumed that carbon metabolism and energy metabolism are separate and share no common intermediates. However, there are suggestions to the contrary (18, 40); acetate kinase, phosphotransacetylase, and CO dehydrogenase have been offered as possible acetate-activating enzymes for energy and carbon metabolism. A defect in a common activation step needed for assimilating exogenous acetate and for methanogenesis from acetate could lead to a mutant defective in both functions. If strain FA^{r}9 is defective in acetate activation, the activation step does not seem to be the formation of acetyl phosphate and acetyl-CoA via acetate kinase and phosphotransacetylase, respectively. The major difference between wild-type and strain FA^{r}9 activities was in phosphotransacetylase. However, the fourfold difference observed between parent and mutant extracts could not account for the 2-order-of-magnitude difference in carbon assimilated. This argument also applies to CO dehydrogenase, which was present at the same specific activity in wild-type and mutant extracts (unless anabolic and catabolic CO dehydrogenase reside on different proteins).

Alternatively, strain FA^{r}9 might be an acetate permeability mutant. If the mutation to FA resistance impaired acetate permeability but not metabolic pathways, strain FA^{r}9 should possess all of the internal enzymes for assimilating acetate into cell carbon and for producing methane. Although our cell extract experiment did not include enzyme assays for methanogenesis from acetate (2, 22), we found substantial levels of activity in mutant extracts for acetate kinase, phosphotransacetylase, and other enzymes capable of converting acetate to TCA intermediates and to phosphoenolpyruvate. Therefore, mutant cell extracts were capable of transforming significantly more acetate than can be accounted for by the quantities of acetate taken up by cells. These results and those of in vivo labeling experiments indicating that acetate kinase and phosphotransacetylase are key enzymes in acetate carbon metabolism (37) suggest that strain FA^{r}9 has a defect in acetate permeation.

Our experiments showing that uptake of acetate at high concentrations was proportional to acetate concentration suggest that diffusion is an important component of acetate uptake. Similar observations and conclusions were reported by Hüster and Thauer (14) for pyruvate uptake in *Methanobacterium thermoautotrophicum*. The proportionality between acetate uptake and acetate concentration was observed for both wild-type and mutant strains, but the quantity taken up was always 20 to 100 times less for the mutant than for the parent strain. Uptake of acetate by the mutant could also be increased by adding NaCN to cultures, although the amount taken up was not sufficient to overcome growth inhibition by NaCN, as it does in wild-type cultures (31). Uptake of propionate by strain FA^{r}9 was not impaired, arguing against a change in the permeability of the membrane itself to the diffusion of small molecules. Permeability to BES and pyruvate was also not altered by the mutation because strain FA^{r}9 did not show increased resistance to growth inhibition. We believe that diffusion of acetate into cells is the principal means of entry, and loss of permeability to acetate suggests that diffusion is facilitated in strain 227 but not in strain FA^{r}9. Because of difficulties inherent in separating internal metabolism from transport, it may not be possible to determine with certainty whether the mutant is defective in acetate permeation or metabolism.

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