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Comparison of Assimilatory Organic Nitrogen, Sulfur, and Carbon Sources for Growth of *Methanobacterium* Species

L. BHATNAGAR,[1] M. K. JAIN,[2] J.-P. AUBERT,[1] AND J. G. ZEIKUS[1]†*

Unité de Physiologie Cellulaire, Departement de Biochemie et de la Génétique Moleculaire, Institut Pasteur, 75015 Paris, France,[1] and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706[2]

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Experiments document the ability of two species of autotrophic methanogens to assimilate and utilize organic substrates as the nutrient sulfur or nitrogen source and as a carbon source during growth on H_2–CO_2. *Methanobacterium thermoautotrophicum* strain ΔH and the mesophilic species *Methanobacterium* sp. strain Ivanov grew with glutamine as the nitrogen source or cysteine as the sulfur source. *M. thermoautotrophicum* also utilized urea as the nitrogen source and as a carbon precursor for methane and cell synthesis. *Methanobacterium* sp. strain Ivanov grew with methionine as the sulfur source. The growth rate of two different *Methanobacterium* species was lower on an organic N or S source than on ammonium or sulfide. ^{35}S and ^{14}C tracer studies demonstrated that amino acid or urea assimilation correlated with time and amount of growth. The rate of [^{35}S]cysteine incorporation was similar in strain ΔH (34 nmol h^{-1} mg of cells^{-1}) and strain Ivanov (23 nmol h^{-1} mg of cells^{-1}). However, the rate of [^{14}C]acetate incorporation was dramatically different (17 versus 208 nmol h^{-1} mg of cells^{-1} in strains ΔH and Ivanov, respectively). [^{14}C]acetate accounted for 1.3 and 21.2% of the total cell carbon synthesized by strains ΔH and Ivanov, respectively. Amino acids and urea were mainly assimilated into the cell protein fraction, but accounted for less than 2.0% of the total cell carbon synthesized. The data suggest that a biochemical-genetic approach to understanding cell carbon synthesis in methanogens is feasible; mutants that are auxotrophic for either acetate, glutamine, cysteine, or methionine are suggested as future targets for genetic studies.

Methanogenic bacteria have been studied for many years, but only recently have techniques been developed to readily grow these fastidious anaerobes on H_2-CO_2 in either liquid culture or on agar plates (1, 3, 4). Thus, present understanding in regard to carbon, sulfur, and nitrogen nutrition and metabolism in methanogens is limited (17); interest in their molecular biology and genetics has just been initiated (13).

Methanogens represent a diverse assemblage of bacteria whose nutritional requirements vary considerably among the different genera (1). *Methanobacterium* species have minimal nutrient requirements and grow autotrophically on H_2-CO_2 with sulfide and ammonium as the sole sources of sulfur and nitrogen (2, 5, 15, 18). Acetate is the only well-documented organic substrate that is assimilated during the growth of *Methanobacterium thermoautotrophicum* on H_2-CO_2 (8, 9). Amino acids play an important role in autotrophic metabolism of *M. thermoautotrophicum* because they are early fixation products of both CO_2 and NH_3 assimilation (8–10).

To develop strategies for the selection of auxotrophic mutants in methanogens, we initiated studies aimed at defining nutrient C, S, and N sources for growth of the autotrophic species *M. thermoautotrophicum* strain ΔH (18) and *Methanobacterium* sp. strain Ivanov (2). Previously, we showed that both the thermophilic and the mesophilic species could utilize elemental sulfur as the sole S source for growth in a medium with 2-mercaptoethanol as reducing agent (4).

The purpose of the present report is to demonstrate that these *Methanobacterium* species are not obligate autotrophs, but they are capable of assimilating acetate, urea, and several amino acids as C, N, or S sources during growth on H_2-CO_2 as the energy source.

(These results were presented elsewhere in preliminary form [M. K. Jain, L. Bhatnagar, J.-P. Aubert, and J. G. Zeikus, p. 525, *In* Abstracts of the Proceedings of the Third International Symposium on Anaerobic Digestion, 14 through 19 August 1983, Boston, Mass.].)

MATERIALS AND METHODS

Chemicals and gases. All chemicals, gases, and reagents used were of analytical grade. Amino acids were obtained from Sigma Chemical Co. (St. Louis, Mo.). All gases were passed over heated copper filings to remove traces of oxygen. Radioactive compounds were obtained from New England Nuclear Corp., Boston, Mass., or Amersham Corp., Arlington Heights, Ill., and had the following specific activities (in millicuries per millimole): 1,2-sodium [^{14}C]acetic acid, 54.7; L-[*U*-^{14}C]glutamine, 281.0; [^{14}C]urea, 7.8; L-[^{35}S]cysteine hydrochloride, 28.6; and, L-[^{35}S]methionine, 189.8.

Organisms and growth conditions. Cultures of *Methanobacterium* sp. strain Ivanov (2) and *Methanobacterium thermoautotrophicum* strain ΔH (18) were grown at 37 or 63°C as described earlier (3). Culture bottles contained 50 to 200 ml of the phosphate-buffered basal minimal medium (PBB medium) described elsewhere (11). This medium contained nitrilotriacetate as a non-metabolizable chelator, but it was prepared and autoclaved without ammonium salts and a sulfur source (11). All carbon, nitrogen, and sulfur sources examined were either autoclaved or filter sterilized (depending on their thermal stability) and added separately by syringe to a 10 mM final concentration. The medium reductant for all nutrient sulfur source determinations was 20 mM 2-mercaptoethanol, whereas, 2 mM Na_2S was used for nutrient nitrogen source determinations. Culture bottles

* Corresponding author.
† Present address: Department of Bacteriology, University of Wisconsin, Madison, WI 53706.

TABLE 1. Comparison of organic substrates as nutrient nitrogen sources for growth of *Methanobacterium* species[a]

Nitrogen source	Final growth yield[b]			
	M. thermoautotrophicum ΔH		*Methanobacterium* sp. strain Ivanov	
	$-NH_4^+$	$+NH_4^+$	$-NH_4^+$	$+NH_4^+$
Glutamine	0.80	0.85	0.97	1.25
Cysteine	0.11	0.83	0.07	0.95
Methionine	0.21	0.78	0.07	0.90
Alanine	0.16	0.83	0.08	0.90
Leucine plus isoleucine	0.14	0.78	0.08	0.90
Aspartate	0.05	0.75	0.10	0.93
Glutamate	0.16	0.74	0.11	1.00
Methylamine	0.19	0.80	0.07	0.95
Urea	0.57	0.82	0.11	0.95
Control	0.05	0.85	0.05	1.10

[a] Anaerobic bottles contained 50 ml of PBB medium, 2.0 mM Na_2S, and 10 mM of the nitrogen source indicated. Cultures were inoculated with control cultures (2.0%) and gassed every 24 h with H_2-CO_2 (80:20) and incubated with shaking at 63°C for *M. thermoautotrophicum* strain ΔH and at 37°C for *Methanobacterium* sp. strain Ivanov. The results represent the averages of three replicate bottles. All experiment results were repeated at least twice.

[b] The results are expressed as optical density maxima at 660 nm.

were inoculated and pressurized daily with 2.5 atm (1 atm = 101.3 kPa) overpressure of H_2-CO_2 (80:20).

Quantification of growth and methanogenesis. Growth was measured by light scattering at 660 nm after insertion of the culture bottle side arm into a spectronic 20 (Bausch & Lomb Inc., Rochester, N.Y.) spectrophotometer. Methane was measured by flame ionization detection after injecting 0.4 ml of culture headspace into a gas chromatograph.

Radiotracer assimilation studies. A ^{14}C- or ^{35}S-labeled organic substrate (20 μCi) was added to a final concentration of 20 mM in culture bottles that contained 200 ml of the medium described above. The medium reductant for all ^{35}S incorporation studies was titanium(III) nitrolotiacetate (11), whereas 2.6 mM Na_2S was used for ^{14}C studies. The bottles were inoculated, gassed to 2.0 atm overpressure H_2-CO_2 (80:20), and incubated with shaking at 37 or 60°C for strain Ivanov or strain ΔH, respectively. Liquid samples (10 ml) were withdrawn by syringe from each of these bottles for analysis at different time intervals and passed through preweighed 0.4-μm filters. The cells on the filter were washed three times with cold carrier solution and then five times with 0.02 M potassium phosphate buffer (pH 7.5). These filters were dried and weighed and then placed into scintillation vials that contained 5 ml of Instagel (Packard Instrument Co., Inc., Rockville, Md.). Radioactivity was then measured in a Packard Tri-Carb liquid scintillation counter. The culture gas phase was analyzed for $^{14}CH_4$ and $^{14}CO_2$ by the gas chromatograph-gas proportional counting method described elsewhere (12). The remaining cells were harvested by centrifugation at the end of growth and fractionated by the procedure of Clark and Schmidt (6) to examine the incorporation of radioactivity into different cellular substituents.

RESULTS

Growth studies. Initial experiments compared the ability of various organic substrates to serve as the nutrient nitrogen source for growth of *M. thermoautotrophicum* and *Methanobacterium* sp. strain Ivanov (Table 1). Controls without ammonium or organic substrates did not display growth. *M. thermoautotrophicum* grew repeatedly (i.e., 10 transfers) with either glutamine or urea, but not with cysteine, methionine, alanine, methylamine, aspartate, leucine plus isoleucine, or glutamate as the sole nitrogen source. On the other hand, *Methanobacterium* sp. strain Ivanov grew repeatedly only with glutamine or ammonium as the sole nutrient nitrogen source. The growth of both species was not significantly inhibited by the sources and concentrations of organic nitrogen tested in control experiments with media that also contained ammonium. Figure 1 compares the time courses for growth and methanogenesis of *Methanobacterium* species grown on organic substrates as the sole nutrient source. The growth rate (0.11 divisions per h) and yield of *Methanobacterium* sp. strain Ivanov were nearly the same on glutamine or ammonium (data not shown) as the sole nitrogen source. However, the growth yield of *M. thermoautotrophicum* was lower on urea than on glutamine or ammonium (0.57 versus 0.80 to 0.85 divisions per h), and the growth rate in divisions per hour decreased from 0.33 on ammonium to 0.2 on urea to 0.17 on glutamine.

Table 2 compares the ability of *Methanobacterium* species to use cysteine or methionine as the nutrient sulfur source

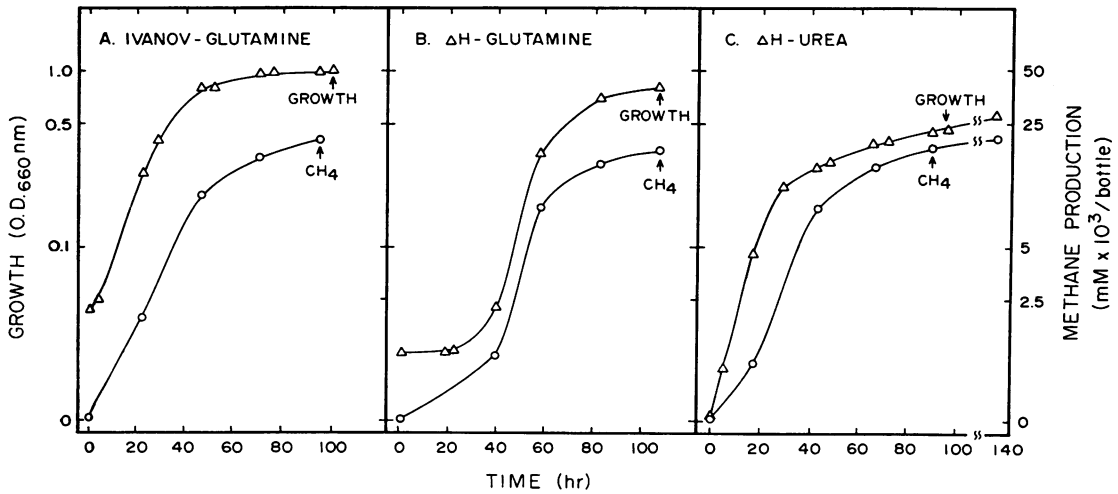

FIG. 1. Fermentation time course of *Methanobacterium* species grown on organic substrates as the nitrogen source. *M. thermoautotrophicum* strain ΔH and *Methanobacterium* sp. strain Ivanov were grown on H_2-CO_2 in culture bottles that contained 50 ml of PBB medium and 10 mM glutamine or urea.

TABLE 2. Comparison of amino acids as nutrient sulfur sources for growth of *Methanobacterium* species[a]

Amino acid	Final growth yield[b]			
	M. thermoautotrophicum ΔH		*Methanobacterium* sp. strain Ivanov	
	$-Na_2S$	$+Na_2S$	$-Na_2S$	$+Na_2S$
Cysteine	0.91	1.00	0.80	1.05
Methionine	0.15	0.87	0.95	1.05
Control	0.10	1.10	0.17	1.10

[a] Anaerobic bottles contained 50 ml of PBB medium, 20 mM 2-mercaptoethanol, and 10 mM amino acid as indicated. Cultures were inoculated with control cultures (2.0%) and gassed every 24 h with H_2-CO_2 (80:20) and incubated with shaking at 63°C for *M. thermoautotrophicum* strain ΔH and at 37°C for *Methanobacterium* sp. strain Ivanov. The results represent the averages of three replicate bottles. All experimental results were repeated at least twice. Cysteine and methionine solutions contained less than 0.0027 mM sulfide.

[b] The results are expressed as optical density maxima at 660 nm.

for growth. *M. thermoautotrophicum* only utilized cysteine, whereas *Methanobacterium* sp. strain Ivanov utilized both amino acids. Figure 2 compares the time courses for growth and methanogenesis of *Methanobacterium* species on cysteine or methionine as the nutrient sulfur source. The growth rate in divisions per hour of *Methanobacterium* sp. strain Ivanov was equivalent (0.03) on both methionine or cysteine, but more rapid with sulfide (0.11) as the nutrient sulfur source. Likewise, the growth rate of *M. thermoautotrophicum* was more rapid on sulfide (data not shown) than cysteine as the nutrient sulfur source (0.33 versus 0.07 divisions per h).

Assimilation studies. In view of discovering that *Methanobacterium* species could utilize various organic substrates as the sole apparent nutrient nitrogen or sulfur sources, experiments (Fig. 3 through 7) were initiated to compare the cellular incorporation kinetics and patterns of the ^{35}S- or ^{14}C-labeled organic compounds under similar growth conditions. In general, incorporation of organic N and S sources paralleled growth and methanogenesis. Thus, the results supported their functions as nutrient S or N sources. As expected (Fig. 3), both *Methanobacterium* strains incorporated

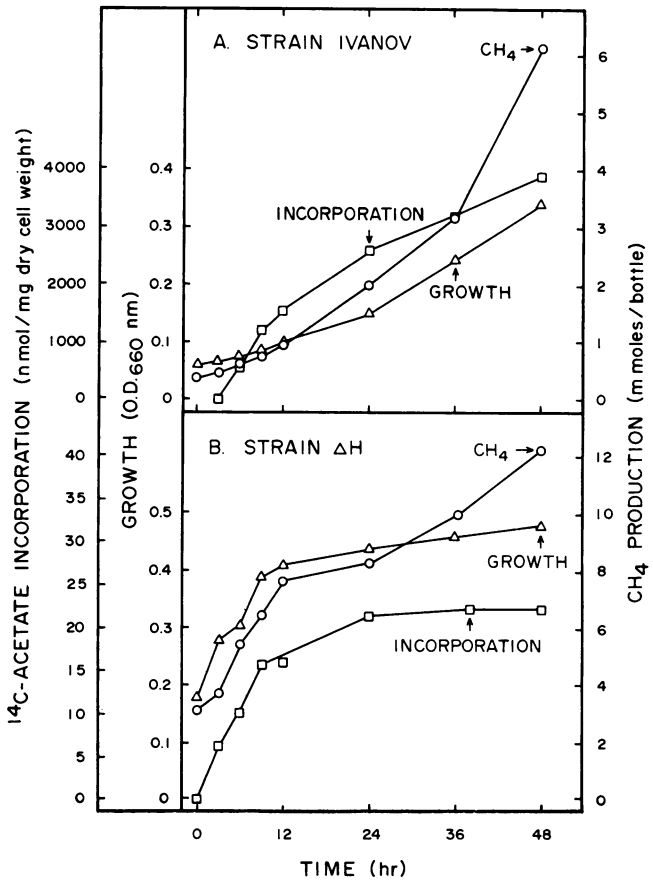

FIG. 3. Comparison of [^{14}C]acetate incorporation during growth of *Methanobacterium* species on H_2-CO_2. *M. thermoautotrophicum* strain ΔH and *Methanobacterium* sp. strain Ivanov were grown in culture bottles that contained 200 ml of PBB medium, 20 mM acetate, 20 μCi of [^{14}C]acetate, 18.7 mM ammonium chloride, and 2.6 mM sodium sulfide.

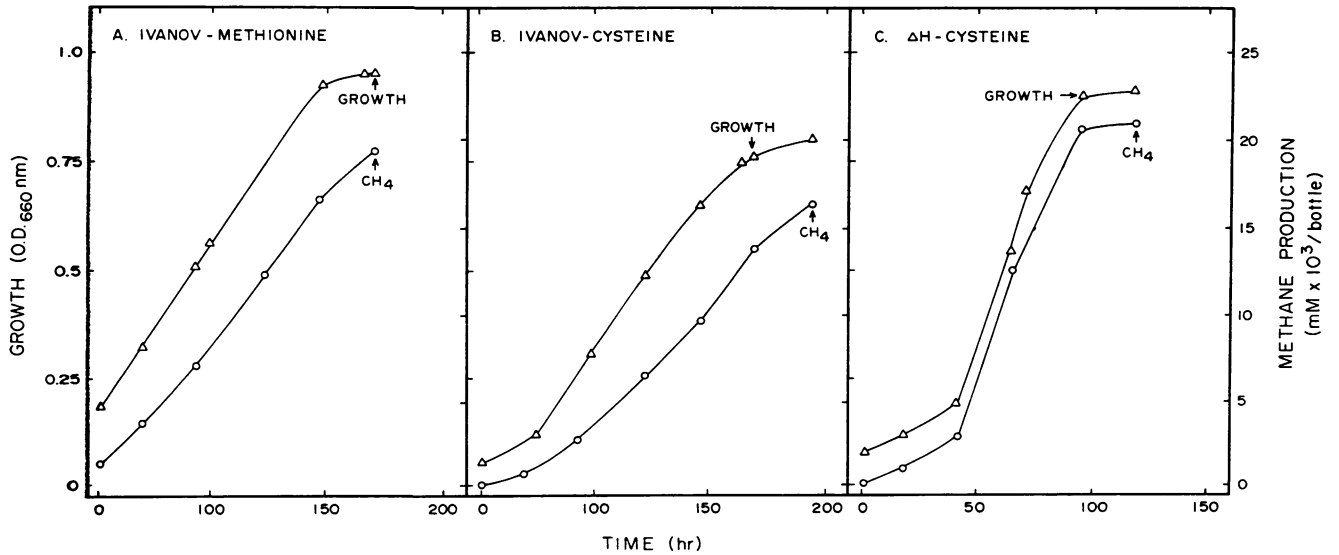

FIG. 2. Fermentation time course of *Methanobacterium* species grown on organic substrates as the sulfur source. *M. thermoautotrophicum* strain ΔH and *Methanobacterium* sp. strain Ivanov were grown on H_2-CO_2 in culture bottles that contained 50 ml of PBB medium, 10 mM methionine or cysteine, and 20 mM 2-mercaptoethanol.

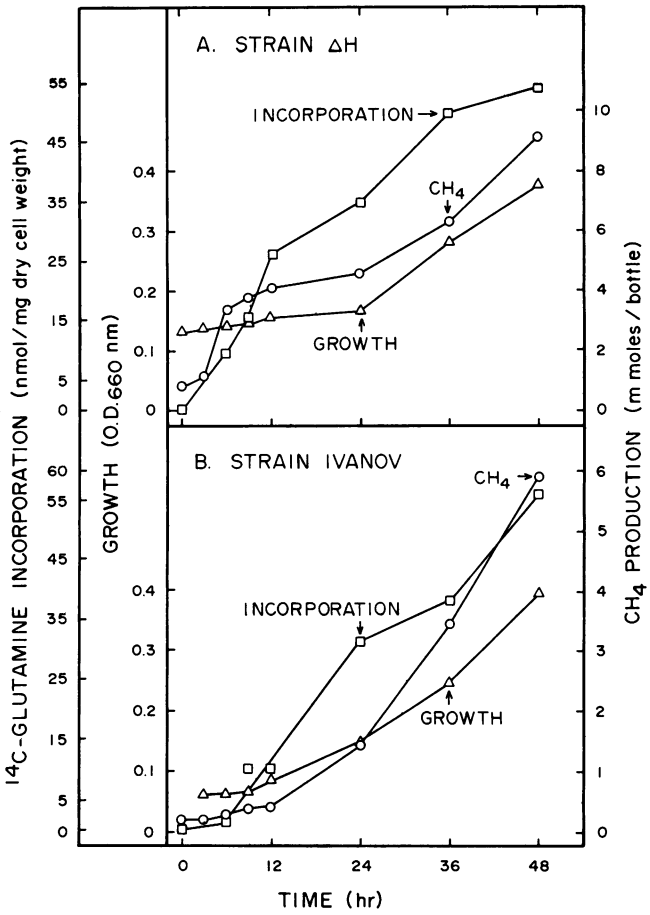

FIG. 4. Comparison of [^{14}C]glutamine incorporation during growth of *Methanobacterium* species on H_2-CO_2. *M. thermoautotrophicum* strain ΔH and *Methanobacterium* sp. strain Ivanov were grown in culture bottles that contained 200 ml of PBB medium, 20 mM glutamine, 20 μCi of [^{14}C]glutamine, and 2.6 mM sodium sulfide.

[^{14}C]acetate, a known carbon precursor for *M. thermoautotrophicum* (8, 9), but the incorporation rate for strain Ivanov (208 nmol h^{-1} mg of $cells^{-1}$) was approximately 10-fold higher than that for strain ΔH (17 nmol h^{-1} mg of $cells^{-1}$). Both strains incorporated [^{14}C]glutamine at similar rates during growth (Fig. 4). *M. thermoautotrophicum* incorporated [^{14}C]urea (5 nmol h^{-1} mg of $cells^{-1}$) at about one-seventh the rate of [^{35}S]cysteine incorporation (Fig. 5 and 6), whereas [^{35}S]methionine (44 nmol h^{-1} mg of $cells^{-1}$) was incorporated at nearly twice the rate of [^{35}S]cysteine by *Methanobacterium* sp. strain Ivanov (Fig. 6 and 7). During growth of *M. thermoautotrophicum* on [^{14}C]urea as the nitrogen source, both $^{14}CO_2$ and $^{14}CH_4$ were detected in the culture headspace. About 1.3% of the total methane formed was derived from urea as determined from specific activity calculations.

Table 3 compares the amount of labeled organic substrate incorporated and its distribution into various cellular components at the end of growth of *M. thermoautotrophicum* or *Methanobacterium* sp. strain Ivanov. The organic nitrogen or sulfur sources tested were similarly distributed into all major cell components. Most of the label was incorporated into the cell protein fraction. However, only acetate assimilation by *Methanobacterium* sp. strain Ivanov accounted for a major contribution to total cell carbon synthesis during growth of *Methanobacterium* species on H_2-CO_2. Acetate accounted for 21.2 and 1.3% of the total cell carbon synthesized by strains Ivanov and ΔH, respectively. Separate experiments performed identically to those presented in Table 3, except that *M. thermoautotrophicum* was grown with [^{14}C]glutamine or [^{35}S]cysteine substrate in the presence of 18.7 mM ammonium or 2.6 mM sulfide, respectively, demonstrated that ammonium decreased total glutamine incorporation and sulfide decreased total cysteine incorporation by 40%.

DISCUSSION

In general, the present data provide the first evidence in support of the following conclusions: autotrophic *Methanobacterium* species can utilize glutamine, methionine, and cysteine as either the apparent sole nutrient, nitrogen, or sulfur source for growth; *Methanobacterium* species differ significantly in their ability to assimilate acetate as a major carbon source during growth on H_2–CO_2; and urea can serve as the nutrient nitrogen source and as a carbon precursor to methane during growth of *M. thermoautotrophicum* on H_2–CO_2. Thus, *Methanobacterium* species, which readily grow on hydrogen, carbon dioxide, sulfide, and ammonium as the respective electron donor, carbon, sulfur, and nitrogen sources are not obligate autotrophs since they display a limited capacity for utilizing certain organic substrates as major carbon, nitrogen, or sulfur precursors.

Under the growth conditions employed here (i.e, pH 7.0 and 20 mM acetate), acetate was a significant precursor for total cell carbon synthesized by *Methanobacterium* sp. strain Ivanov, but not *M. thermoautotrophicum* strain ΔH. Previous studies (9) demonstrated that lower pH values and

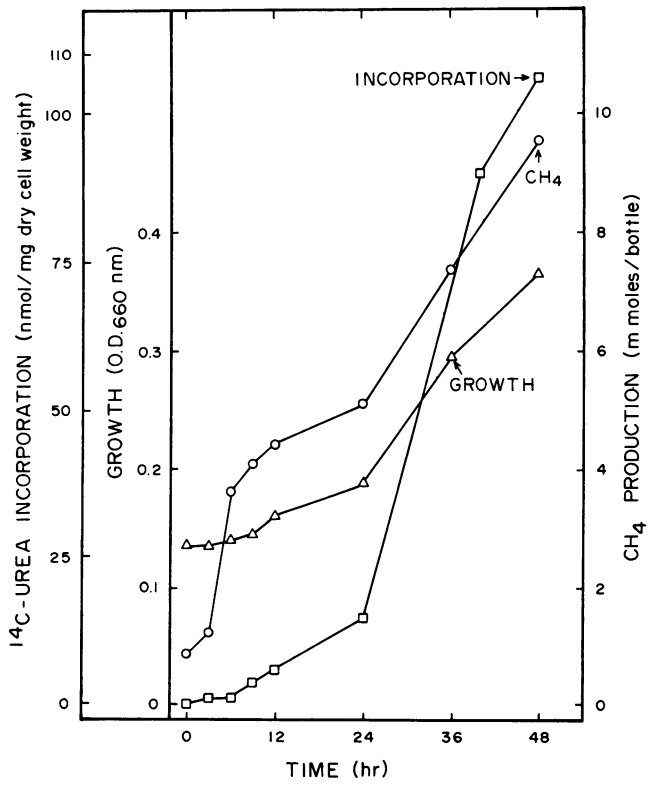

FIG. 5. Incorporation of [^{14}C]urea during growth of *M. thermoautotrophicum* strain ΔH on H_2-CO_2. Culture bottles contained 200 ml of PBB medium, 20 mM urea, 20 μCi of [^{14}C]urea, and 2.6 mM sodium sulfide.

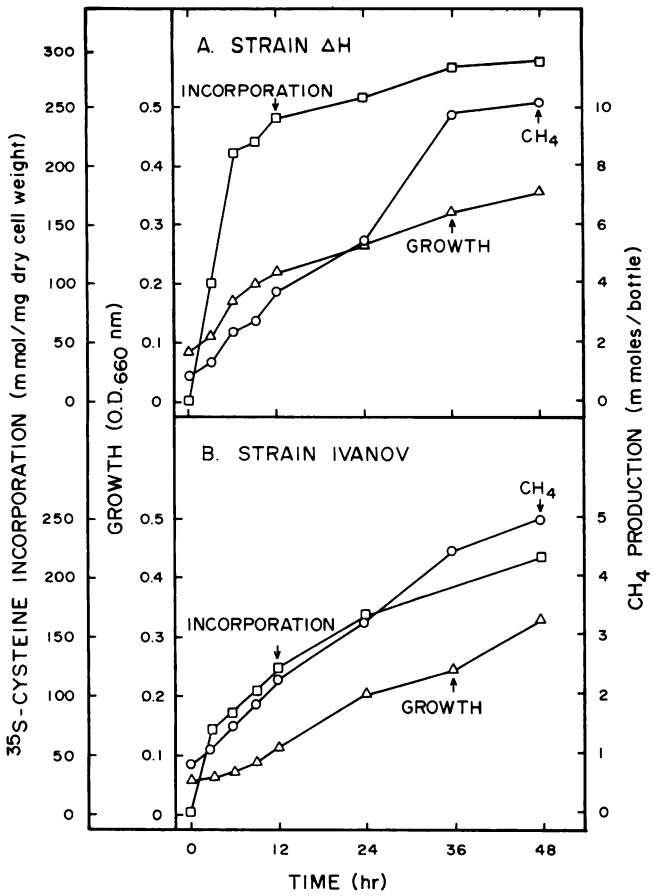

FIG. 6. Comparison of [^{35}S]cysteine incorporation during growth of *Methanobacterium* species on H_2-CO_2. *M. thermoautotrophicum* strain ΔH and *Methanobacterium* sp. strain Ivanov were grown in culture bottles that contained 200 ml of PBB medium, 20 mM cysteine, 20 μCi of [^{35}S]cysteine, 18.7 mM ammonium chloride, and 0.3 to 0.5 ml of titanium(III) nitrilotriacetate.

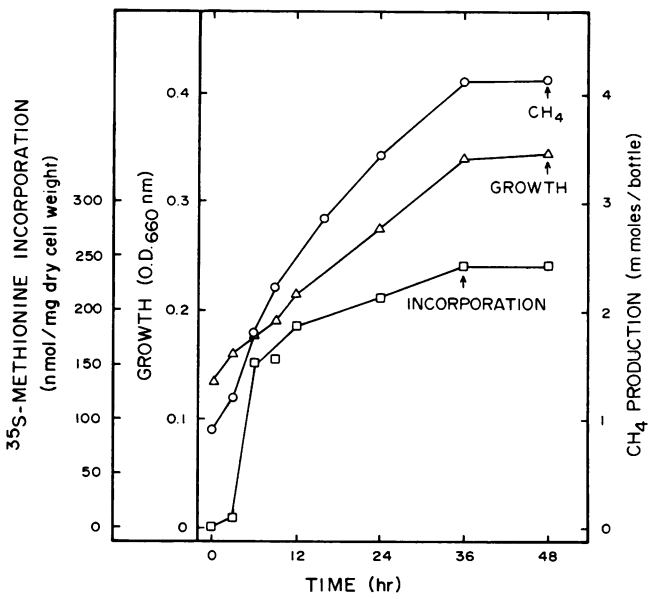

FIG. 7. Incorporation of [^{35}S]methionine during growth of *Methanobacterium* sp. strain Ivanov on H_2-CO_2. Culture bottles contained 200 ml of PBB medium, 20 mM methionine, 20 μCi of [^{35}S]methionine, 18.7 mM ammonium chloride, and 0.5 ml of titanium(III) nitrilotriacetate.

higher acetate concentrations were required for significant acetate assimilation in *M. thermoautotrophicum* strain Marburg, which suggested that acetate was passively assimilated as the non-dissociated form of the acid. Thus, the present results imply, but do not prove, that *Methanobacterium* sp. strain Ivanov may have an active uptake mechanism for acetate which is not comon to *M. thermoautotrophicum* strain ΔH. The *Methanobacterium* species examined differ from some *Methanococcus* (2) and *Methanobrevibacterium* (1, 5) species that require high acetate concentrations for growth.

These *Methanobacterium* species prefer ammonium as the nitrogen source and sulfide as sulfur source rather than glutamine and methionine or cysteine, respectively, because the growth rate was not enhanced when amino acids served as the sole nitrogen or sulfur sources, and the presence of ammonium or sulfide decreased the incorporation of the respective amino acids into cell materials. Methionine was previously reported to serve as a sulfur source for *Methanosarcina barkeri* (14); however, only

TABLE 3. Amount and distribution of radioactivity incorporated into cellular components after growth of methanogens on H_2-CO_2 with organic additions[a]

Strain	Substrate	Total incorporated (nmol/mg of cell wt)	Component (% distribution)			
			Soluble pool and low-molecular-wt intermediates	Lipids	Nucleic acids	Protein and cell residue
M. thermoautotrophicum ΔH	[^{14}C]acetate	235	4.3	9.4	19.9	66.4
	[^{14}C]glutamine	53	34.8	11.9	12.7	40.6
	[^{14}C]urea	106	11.0	17.8	18.1	53.1
	[^{35}S]cysteine	271	12.1	23.8	23.2	40.8
Methanobacterium sp. strain Ivanov	[^{14}C]acetate	3,883	2.8	4.8	30.7	61.6
	[^{14}C]glutamine	56	38.6	13.0	12.6	35.8
	[^{35}S]cysteine	215	22.1	22.2	16.5	39.2
	[^{35}S]methionine	243	16.2	17.8	18.8	47.2

[a] Anaerobic bottles contained 200 ml of PBB medium and 20 mM of the substrate (20 μCi) indicated as either the sole N or S source, except for acetate studies, which contained 18.7 mM ammonium and 2.6 mM sulfide. Bottles were gassed every 24 h to 2 atm overpressure with H_2-CO_2 (80:20) and incubated with shaking at 60°C for strain ΔH and at 37°C for strain Ivanov. The total substrate incorporation reported was calculated at 48 h after the addition of radioactive compounds. The remaining cells were centrifuged at 11,000 × *g* and washed three times with 0.02 M phosphate buffer (pH 7.5) before the distribution of radioactivity in cellular material was examined (7). All experiments were repeated at least two to three times with similar results.

Methanobacterium sp. strain Ivanov used this amino acid. It appears that substrate uptake is a rate-limiting step in the utilization of organic substrates by *Methanobacterium* species grown on H_2–CO_2 because the rate of amino acid assimilation was lower than that of acetate, except for cysteine uptake by strain ΔH. Amino acid metabolism of the *Methanobacterium* species examined also differed from that of *Methanococcus voltae*, which requires isoleucine and leucine for growth (16).

The discovery that *M. thermoautotrophicum* used urea as a carbon and nitrogen source is of considerable interest because it suggests that methanogens may contain the nickel enzyme urease, which is present in other bacterial trophic groups found in anaerobic manure digestors. Alternatively, urea could be a substrate for CO dehydrogenase, which is present in methanogens; the function of this nickel enzyme is not proven, but it is suggested to be involved with acetate metabolism (17). Previously, biochemical-genetic approaches to understanding cell carbon synthesis in methanogens were not considered favorable because the obligate nature of autotrophic species suggested they were not able to assimilate organic C, S, or N sources.

The present data suggest several strategies for obtaining metabolic mutants and initiating classic genetic studies with *Methanobacterium* species. First, *Methanobacterium* sp. strain Ivanov appears as better a model organism than *M. thermoautotrophicum* for selection of auxotrophic mutants because it assimilates more amino acids, it incorporates acetate more rapidly, and it forms large colonies on agar at 37°C, which facilitates replica plating. Thus, future studies will be aimed at obtaining metabolic mutants of *Methanobacterium* sp. strain Ivanov that are auxotrophic for methionine, cysteine, glutamine, and acetate to better understand sulfur, nitrogen, and carbon metabolism in autotrophic methanogens.

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