

Effect of Dilution Rate on Metabolic Pathway Shift between Aceticlastic and Nonaceticlastic Methanogenesis in Chemostat Cultivation

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Acetate conversion pathways of methanogenic consortia in acetate-fed chemostats at dilution rates of 0.025 and 0.6 day⁻¹ were investigated by using ¹³C-labeled acetates, followed by gas chromatography-mass spectrometry (GC-MS) analysis of the CH₄ and CO₂ produced. Nonaceticlastic syntrophic oxidation by acetate-oxidizing syntrophs and hydrogenotrophic methanogens was suggested to occupy a primary pathway (approximately 62 to 90%) in total methanogenesis at the low dilution rate. In contrast, aceticlastic cleavage of acetate by aceticlastic methanogens was suggested to occupy a primary pathway (approximately 95 to 99%) in total methanogenesis at the high dilution rate. Phylogenetic analyses of transcripts of the methyl coenzyme M reductase gene (*mcrA*) confirmed that a significant number of transcripts of the genera *Methanoculleus* (hydrogenotrophic methanogens) and *Methanosarcina* (aceticlastic methanogens) were present in the chemostats at the low and high dilution rates, respectively. The *mcrA* transcripts of the genus *Methanosaeta* (aceticlastic methanogens), which dominated the population in a previous study (T. Shigematsu, Y. Tang, H. Kawaguchi, K. Ninomiya, J. Kijima, T. Kobayashi, S. Morimura, and K. Kida, *J. Biosci. Bioeng.* 96:547-558, 2003), were poorly detected at both dilution rates due to the limited coverage of the primers used. These results demonstrated that the dilution rate could cause a shift in the primary pathway of acetate conversion to methane in acetate-fed chemostats.

Under methanogenic conditions, acetate is quantitatively the most dominant intermediate of anaerobic degradation of organic matter. It is estimated that approximately 70 to 80% of methane is derived from acetate in anoxic environments (11, 13, 14). Two processes by which acetate is converted to methane have been described. The acetate-utilizing methanogens, the genera *Methanosaeta* and *Methanosarcina*, use the aceticlastic cleavage pathway in which the methyl group of acetate is converted to methane, while the carboxyl group is converted to CO₂ (4). The second process includes the syntrophic oxidation of acetate to CO₂ and hydrogen by one organism and the subsequent reduction of carbon dioxide to methane by a hydrogenotrophic methanogen. Two thermophilic bacteria, strain AOR and *Thermacetogenium phaeum*, and one mesophilic bacterium, *Clostridium ultunense*, were demonstrated to be capable of acetate oxidation in cocultures with hydrogenotrophic methanogens (7, 10, 19). The net reaction is the same as the reaction for aceticlastic cleavage of acetate in the syntrophic acetate oxidation process, but ¹⁴C-labeled substrates have been used to differentiate between the two processes (16, 18, 24). The quantitative information for these two acetate conversion pathways in total methanogenic microbial communities has been limited to date.

In a previous study, chemostat cultures of mesophilic ace-

tate-degrading methanogenic consortia were constructed (8). The relative concentration of coenzyme F₄₂₀, which is involved in hydrogenotrophic methanogenesis, was much higher at low dilution rates than that at high dilution rates. Microbial community structure analysis of the chemostat cultures at dilution rates of 0.025 and 0.6 day⁻¹ revealed that a significant number of cells of the genus *Methanoculleus*, which is a hydrogenotrophic methanogen, were detected only at the low dilution rate, although larger populations of aceticlastic methanogens affiliated with the genera *Methanosaeta* and *Methanosarcina* were detected at both dilution rates (21). The detection of hydrogenotrophic methanogens and higher F₄₂₀ concentrations in the chemostat cultures at the low dilution rate suggests that a significant proportion of methanogenesis occurs by syntrophic acetate oxidation rather than by aceticlastic cleavage of acetate. In this study, we analyzed the acetate conversion pathways of methanogenic consortia using ¹³C-labeled substrates and gas chromatography-mass spectrometry (GC-MS) analysis of the CH₄ and CO₂ produced. We also performed a phylogenetic analysis of transcripts of the *mcrA* gene, which encodes the α -subunit of methyl coenzyme M reductase I (MCR I), at both dilution rates.

MATERIALS AND METHODS

Operation of acetate-fed chemostats. Two anaerobic chemostats were operated for more than 2 years at 37°C with acetate as the only added carbon source and electron donor at dilution rates of 0.025 and 0.6 day⁻¹. Completely stirred tank reactors, each with a working volume of 1.7 liters, were used as the chemostats. The inoculum for these reactors was digested sludge acclimatized in our laboratory for 6 months. Detailed operation methods have been described previously (8). Under steady-state conditions, the acetate concentrations in the

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chemostats were approximately 10 and 250 mg/liter at dilution rates of 0.025 and 0.6 day⁻¹, respectively.

Batchwise gas evolution test with ¹³C-labeled substrate. A 10-ml sample of culture broth was taken from a chemostat and centrifuged at 25,000 × g for 15 min. The precipitate was washed with 30 ml of synthetic wastewater (8) containing no carbon source and was resuspended in 10 ml of synthetic wastewater containing no carbon source. The precipitate was transferred to vials and supplemented with [1-¹³C]sodium acetate, [2-¹³C]sodium acetate, or [1,2-¹³C]sodium acetate to give a final concentration of 100 mM. Washing and suspension of the pellet were performed by using an anaerobic glove box (model 1025; Forma Scientific, Marietta, Ohio) supplemented with helium gas. The vials were immersed in a thermostat-controlled water bath at 37°C. After 12 h of incubation with mixing, the CH₄ and CO₂ in the headspace were analyzed by using a GCMS-QP5000 GC-MS (Shimadzu, Kyoto, Japan) equipped with a GS-GasPro column (30 m by 0.32 mm; J & W Scientific, Folsom, Calif.). Helium was used as carrier gas at a flow rate of 1.7 ml/min. The column temperature was 30°C. The peaks at *m/z* 15 and 17 in the mass spectrum, which were derived from a retention time of 1.2 min in the gas chromatogram, were regarded as the fragment ion for ¹²CH₄ and the molecular ion for ¹³CH₄, respectively. The peaks at *m/z* 44 and 45, which were derived from a retention time of 1.5 min, were regarded as the molecular ions for ¹²CO₂ and ¹³CO₂, respectively.

RT-PCR amplification and cloning. Total nucleic acids from the culture broth in a chemostat were extracted by a method described previously (21). RNA was then extracted by the method of Griffiths et al. (5) and purified with an RNeasy kit (QIAGEN, Hilden, Germany). Reverse transcription (RT) reactions were performed with a Gene Amp Gold RNA PCR reagent kit (Applied Biosystems, Foster City, Calif.) by using 500 and 100 ng of the template RNA extracted from the chemostats at dilution rates of 0.025 and 0.6 day⁻¹, respectively, and the reverse primer ME2 (5'-TCAT(G/T)GC(A/G)TAGTT(A/G/T)GG(A/G)TAG T-3') (6). The resulting cDNA was purified with a MicroSpin S-400 HR column (Amersham Biosciences, Piscataway, N.J.) and was used as the template for amplification of *mcrA* with AmpliTaq (Applied Biosystems, Foster City, Calif.) used according to the manufacturer's instructions (1× PCR buffer, 2.5 U of AmpliTaq DNA polymerase, each deoxynucleoside triphosphate at a concentration of 250 μM, and 40 pmol of each primer in a 100-μl reaction mixture). The PCR primers used in the amplification were the forward primer ME1 (5'-GC(A/C)ATGCA(A/G)AT(A/C/T)GG(A/T)ATGTC-3') (6) and the reverse primer ME2b (5'-TCCTG(G/C)AGGTCG(A/T)A(A/G)CCGAAGAA-3'). Reactions were performed with a GeneAmp PCR System 2400 (Applied Biosystems) with the following cycle conditions: preincubation at 95°C for 2 min; 25 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. The amplified *mcrA* fragments were cloned into a plasmid pT7Blue T vector (Novagen Inc., Madison, Wis.) by using a DNA ligation kit (version 2; Takara, Kyoto, Japan).

Sequencing and phylogenetic analysis. Cloned RT-PCR products were prepared from randomly selected recombinants and used as templates for sequencing. Sequencing was performed by using a DNA sequencer (CEQ8000; Beckman Coulter, Fullerton, Calif.) with a CEQ Quick Start Master Mix kit (Beckman Coulter). DNA and deduced amino acid sequences were analyzed with the GENETYX-WIN software package (version 5.1; Software Development, Tokyo, Japan). The search for homologous proteins was conducted with the BLAST program (1). Multiple alignments were run by using the Clustal X program, version 1.8 (22). Phylogenetic analyses were conducted with MEGA, version 2.1 (9). Identical sequences (100% similarity) were recognized by matrix analysis and manual comparison and were used in subsequent analyses as an operational taxonomic unit (OTU). The OTUs were designated ALM01 to ALM08 for clones from the culture broth at a dilution rate of 0.025 day⁻¹ and AHM01 to AHM08 for clones at a dilution rate of 0.6 day⁻¹.

Quantitative RT-PCR of *mcrA* transcripts. Real-time quantitative RT-PCR experiments were conducted to quantify *mcrA* transcripts of different taxonomic groups by using the TaqMan fluorogenic PCR system. The RT reaction was performed with a Gene Amp Gold RNA PCR reagent kit (Applied Biosystems) by using 10 μg of the extracted RNA as a template and the reverse primer ME2 in a 100-μl reaction mixture. The resulting cDNA was precipitated by ethanol precipitation and then vacuum dried and resuspended in 20 μl of Tris-EDTA buffer (pH 7.4). The cDNA was purified by using a MicroSpin S-400 HR column (Amersham Biosciences) and was then used as a template for a quantitative PCR. The quantitative PCR was carried out by using primers ME1 and ME2b and a genus-specific TaqMan probe. Three TaqMan probes, SAE716TAQ (5'-AGGCCTTCCCCACTCTGCTTGAGGAT-3'), SAR716TAQ (5'-AGAAATCCCAACAGCCCTTGAAGAC-3'), and MCU716TAQ (5'-AGCAGTACCCGACCATGATGGAGGAC-3'), were used for detection of the *mcrA* gene products of the genera *Methanosaeta*, *Methanosarcina*, and *Methanoculleus*, respec-

tively. The specificities of these probes for the target *mcrA* genes were confirmed by manual comparison of the nucleotide sequences of *mcrA* genes in the DDBJ/EMBL/GenBank database. All TaqMan probes were 5' end labeled with 6-carboxyfluorescein and 3' end labeled with 6-carboxytetramethyl rhodamine, obtained from Applied Biosystems. In order to evaluate the selectivity of the primer-probe sets, three clones, ALM07, AHM01, and ALM01, were used as controls. By using the three sets of primers and TaqMan probes, fluorescence signal monitoring was performed with the GeneAmp 5700 sequence detection system (Applied Biosystems). Reaction mixtures for fluorogenic PCR in which the concentrations of both the primer and the TaqMan probe were optimized (300 and 200 nM, respectively) were prepared. The concentration of control DNA varied between 15.63 and 625 pg per 50 μl of reaction mixture. The TaqMan Universal PCR Master Mix (Applied Biosystems) was used with the following cycle conditions: an initial step of 50°C for 2 min and then 95°C for 10 min; and two-step cycles of 95°C for 15 s and 60°C for 1 min. All assays were performed at least in duplicate. Post-PCR analysis was performed by using GeneAmp 5700 SDS software.

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers for the sequences of clones ALM01 to ALM08 and AHM01 to AHM08 are AB158524 to AB158539.

RESULTS

Batchwise gas evolution test with ¹³C-labeled substrates.

Two acetate-fed mesophilic anaerobic chemostats were operated for more than 2 years at dilution rates of 0.025 and 0.6 day⁻¹. Under steady-state conditions, the acetate concentrations in the chemostats were approximately 0.2 and 4 mM at dilution rates of 0.025 and 0.6 day⁻¹, respectively. To evaluate the acetate conversion pathway in the two chemostats, batchwise cultivation of culture broth extracted from the chemostats was carried out by using forms of ¹³C-labeled sodium acetate as the substrates. If the culture broth used aceticlastic cleavage of acetate, the methyl and carboxyl groups of acetate would have been converted to methane and CO₂, respectively (4), but if the culture broth used syntrophic acetate oxidation, 2 mol of CO₂ would have been produced from 1 mol of acetate, while 1 mol of the CO₂ produced would have been concurrently reduced to methane (24). In the latter case, the methyl and carboxyl groups of acetate would have been converted to equal amounts of methane and CO₂.

When the culture broth of the chemostat at a dilution rate of 0.025 day⁻¹ was used for batch cultivation, 33 and 45% of the methane were considered to be derived from the carboxyl group of acetate when [2-¹³C]sodium acetate and [1-¹³C]sodium acetate, respectively, were used as the substrates (Table 1). For CO₂, 31 and 32% were considered to be derived from the methyl group when [2-¹³C]sodium acetate and [1-¹³C]sodium acetate, respectively, were used as the substrates (Table 2). On the other hand, only about 2% of the methane and 6% of the CO₂ were considered to be derived from the carboxyl and methyl groups of acetate, respectively, when the culture broth of the chemostat at a dilution rate of 0.6 day⁻¹ was used. These results suggested that the syntrophic oxidation pathway accounted for approximately 62 to 90% of the total methanogenesis in the chemostat at the low dilution rate. In contrast, at the high dilution rate, the aceticlastic cleavage of acetate was suggested to account for 95 to 99% of total methanogenesis. Because we used batchwise cultivation for the ¹³C-labeled substrate assay, the results might not precisely reflect the in situ activities of the microorganisms in the chemostats. But the culture broth at the low dilution rate had an obviously larger potential for syntrophic acetate oxidation than for aceticlastic

TABLE 1. GC-MS analysis of CH₄ produced from ¹³C-labeled acetate

Dilution rate (day ⁻¹)	Substrate	Peak intensities			CH ₄ from carboxyl base/total CH ₄	CH ₄ from methyl base/total CH ₄
		<i>m/z</i> 15 (¹² CH ₄)		<i>m/z</i> 17 (¹³ CH ₄) (actual)		
		Actual	Background subtracted ^a			
0.025	¹³ CH ₃ ¹² COONa	2,695 ^b	2,695	5,474	0.33	0.67
	¹² CH ₃ ¹³ COONa	1,139	1,139	928	0.45	0.55
	¹³ CH ₃ ¹³ COONa	0		1,361		
0.6	¹³ CH ₃ ¹² COONa	81,973	14,066	614,114	0.022	0.98
	¹² CH ₃ ¹³ COONa	636,401	562,089	13,860	0.024	0.98
	¹³ CH ₃ ¹³ COONa	74,312		588,433		

^a The peak intensities at an *m/z* value of 15 from ¹³CH₃¹³COONa were regarded as the background.

^b All values are averages for duplicate experiments.

cleavage of acetate, whereas the culture broth at the high dilution rate had a larger potential for acetoclastic cleavage than for syntrophic acetate oxidation.

Analysis of *mcrA* gene transcripts from the chemostats. MCR I appears to be unique to methanogens and to be present in all methanogens. The *mcrA* gene encoding the α -subunit of MCR I has been used a marker gene for the specific detection of methanogens in various environments (6, 12). The *mcrA* transcripts in community RNA extracted from the acetate-fed chemostats were amplified by RT-PCR and were used to construct two clone libraries, designated ALM (*mcrA* transcripts from the chemostat at a dilution rate of 0.025 day⁻¹) and AHM (*mcrA* transcripts from the chemostat at a dilution rate of 0.6 day⁻¹). Twenty-one clones from each library were randomly selected and sequenced. All nucleotide sequences and deduced amino acid sequences showed significant similarities with sequences of known *mcrA* genes and MCRs, respectively. In the ALM library, eight different sequences (OTUs) were obtained. Six OTUs (ALM01 to ALM06, 17 clones) were closely related to the *mcrA* gene of *Methanoculleus thermophilicus* (Fig. 1) and were regarded as *mcrA* genes of the genus *Methanoculleus* (Table 3). The other two OTUs (ALM07 and ALM08, four clones) were closely related to the *mcrA* gene of *Methanosaeta concilii* and were regarded as *mcrA* genes of the genus *Methanosaeta*. In the AHM library, eight OTUs were obtained. Seven OTUs (AHM01 to AHM07, 20 clones) were closely related to the *mcrA* gene of *Methanosarcina mazei* and were regarded as *mcrA* genes of the genus *Methanosarcina*.

Another OTU (AHM08, one clone) was closely related to *mcrA* of *M. concilii* and was regarded as an *mcrA* gene of the genus *Methanosaeta*.

Real-time quantitative RT-PCR experiments were conducted to quantify *mcrA* transcripts of different taxonomic groups. *mcrA* transcripts of the genus *Methanosaeta* could not be detected in the RNA from the chemostat at either dilution rate (Table 3). *mcrA* transcripts of the genus *Methanosarcina* could be detected only in the RNA from the chemostat at a dilution rate of 0.6 day⁻¹. In contrast, *mcrA* transcripts of the genus *Methanoculleus* could be detected only in the RNA from the chemostat at a dilution rate of 0.025 day⁻¹. These results agreed with our clonal sequence analysis which showed that the *mcrA* transcripts of the genera *Methanoculleus* and *Methanosarcina* were dominant in RNA from the chemostats at the low dilution rate and the high dilution rate, respectively. Because the ME1 and ME2 primers were reported not to be suitable for amplification of the *mcrA* genes of the genus *Methanosaeta* (12), the selectivity of the primer sets should be considered. However, our results still indicated that the levels of *mcrA* transcription activity of the genera *Methanoculleus* and *Methanosarcina* were significant in the chemostats at the low and high dilution rates, respectively.

DISCUSSION

We used a stable-isotope technique to analyze the primary pathway of acetate conversion to methane in acetate-fed che-

TABLE 2. GC-MS analysis of CO₂ produced from ¹³C-labeled acetate

Dilution rate (day ⁻¹)	Substrate	Peak intensities			CO ₂ from methyl base/total CO ₂	CO ₂ from carboxyl base/total CO ₂
		<i>m/z</i> 44 (¹² CO ₂)		<i>m/z</i> 45 (¹³ CO ₂) (actual)		
		Actual	Background subtracted ^a			
0.025	¹³ CH ₃ ¹² COONa	4,041 ^b	2,635	1,196	0.31	0.69
	¹² CH ₃ ¹³ COONa	3,424	2,018	4,296	0.32	0.68
	¹³ CH ₃ ¹³ COONa	1,406		3,380		
0.6	¹³ CH ₃ ¹² COONa	87,919	79,915	5,302	0.062	0.94
	¹² CH ₃ ¹³ COONa	12,951	4,947	80,702	0.058	0.94
	¹³ CH ₃ ¹³ COONa	8,004		73,096		

^a The peak intensities at an *m/z* value of 44 from ¹³CH₃¹³COONa were regarded as the background.

^b All values are averages for duplicate experiments.

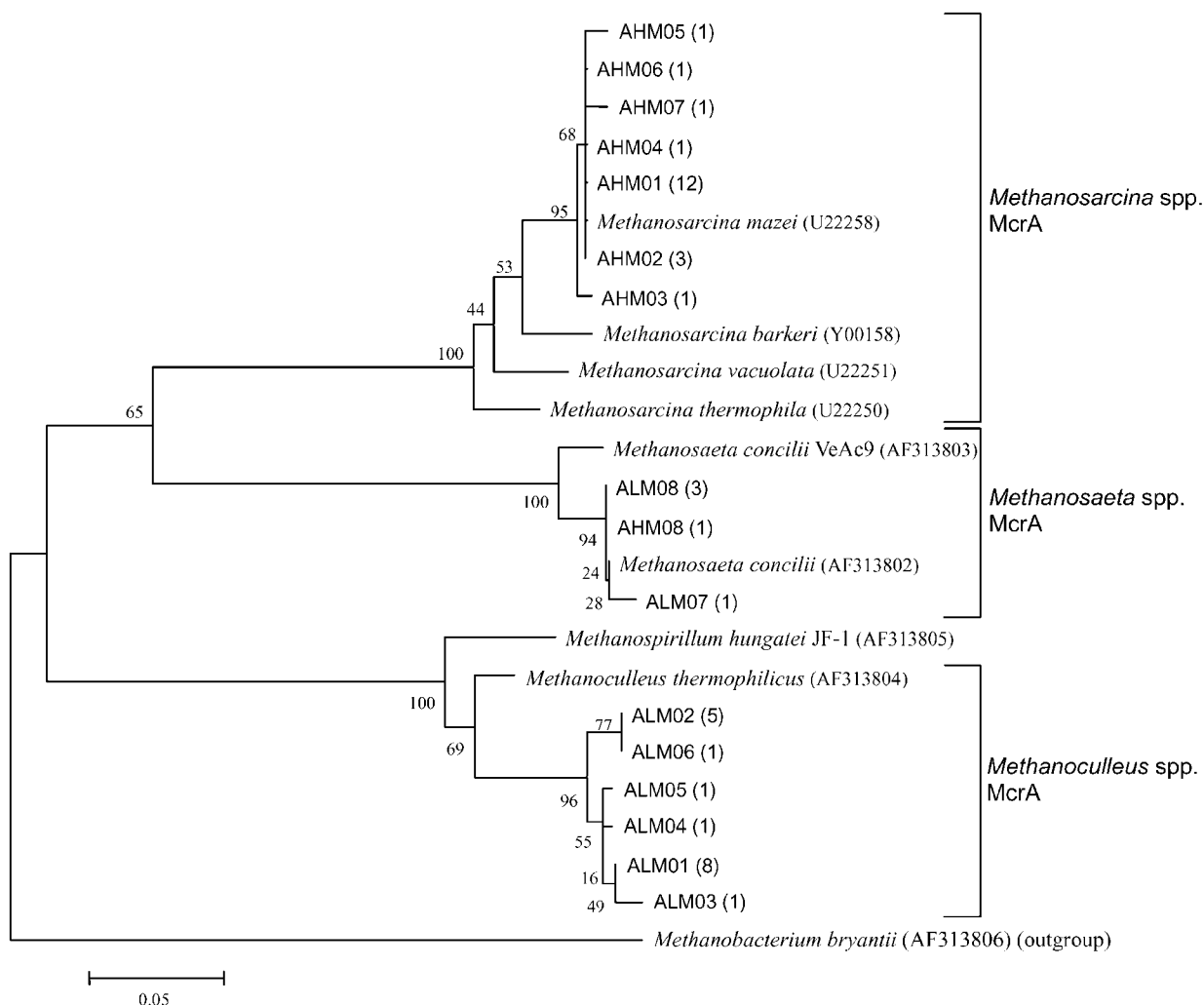


FIG. 1. Phylogenetic relationships of deduced amino acid sequences of α-subunits of MCR I (McrA). The tree was constructed from phylogenetic distances obtained by the neighbor-joining method (17). ALM and AHM indicate clones from cultivation at the low (0.025 day⁻¹) and high (0.6 day⁻¹) dilution rates, respectively. The numbers of clones that had identical sequences are shown in parentheses. Bar = 5 amino acid substitutions per 100 amino acids. Bootstrap probabilities (3) are indicated at branch nodes. The DDBJ/EMBL/GenBank accession numbers for reference strains are shown in parentheses. The tree was rooted by using McrA of *Methanobacterium bryantii* as the outgroup.

mostats. To our knowledge, this was the first application of ¹³C-labeled substrates combined with GC-MS analysis of the methane and CO₂ produced to analyze acetate conversion pathways to methane. This technique is more useful and convenient for determining which groups of acetate are converted to methane and CO₂ than the conventional technique using ¹⁴C-labeled substrates. The results obtained in the RT-PCR experiment targeting *mcrA* transcripts supported the findings obtained by using ¹³C-labeled substrates, although the limited coverage of the primers used requires further consideration.

The specific growth rate of the mesophilic acetate-oxidizing syntroph *C. ultunense* cocultured with a hydrogenotrophic methanogen by using acetate as a substrate was reported to be 0.027 to 0.035 day⁻¹ (20). The specific growth rates of the mesophilic aceticlastic methanogens *M. concilii* and *M. mazei* were 0.24 to 0.28 and 0.98 day⁻¹, respectively (2). At the high dilution rate (0.6 day⁻¹), the cells affiliated with the genus *Methanosarcina*, which were able to grow rapidly, would have

TABLE 3. Composition of and quantification of *mcrA* transcripts of three taxonomic groups

Phylogenetic affiliation ^a	Dilution rate of 0.025 day ⁻¹		Dilution rate of 0.6 day ⁻¹	
	No. of clones	Quantitative RT-PCR (copies/μg of total RNA) ^b	No. of clones	Quantitative RT-PCR (copies/μg of total RNA) ^b
<i>Methanosaeta</i> spp. <i>mcrA</i>	4	ND ^c	1	ND
<i>Methanosarcina</i> spp. <i>mcrA</i>	0	ND	20	4.15 × 10 ⁷
<i>Methanoculleus</i> spp. <i>mcrA</i>	17	9.06 × 10 ⁶	0	ND

^a Primers ME1 and ME2 used in this study were reported not to be suitable for amplification of the *mcrA* genes of the genus *Methanosaeta* (12).

^b The values for quantitative RT-PCR are averages for duplicate experiments.

^c ND, not detected.

been predominant in the chemostat and engaged in aceticlastic cleavage of acetate to methane as the primary pathway. On the other hand, the low dilution rate (0.025 day^{-1}) was sufficiently low for growth of the three acetate-utilizing members, acetate-oxidizing syntrophs, *Methanosaeta*, and *Methanosarcina*. In this case, competition among the substrate affinities of the three acetate-utilizing members would have been decisive for dominance. The apparent K_m for acetate of a thermophilic acetate-oxidizing syntroph was reported to be 0.65 mM (16), although no K_m value of a mesophilic acetate-oxidizing syntroph is currently available. The apparent K_m values for acetate of the genera *Methanosaeta* and *Methanosarcina* were approximately 0.8 to 0.9 and 3 to 5 mM, respectively (23). The acetate-oxidizing syntroph associated with *Methanoculleus*, according to the high substrate affinity, was better adapted to convert acetate primarily in the chemostat at a low dilution rate. The genus *Methanosaeta*, whose population was previously shown to be the largest among the three members by previous ribosomal DNA analyses (21), played a secondary role for acetate conversion by aceticlastic cleavage in the chemostat at the low dilution rate. It is possible that even cells of a dominant population could not show a dominant metabolic function in a consortium because of their lower metabolic activity. The correlation between the population dominance of the genus *Methanosaeta* and its lower metabolic activity for acetate conversion at the low dilution rate still requires further analysis in terms of quantification and comparison of *mcrA* transcripts of the genera *Methanosaeta* and *Methanoculleus* with more universal primers. We have no direct evidence of which bacteria are responsible for acetate oxidation in our chemostats. However, the dominance of bacteria belonging to the phylum *Firmicutes*, with which *C. ultunense* and *T. phaeum* are affiliated, was shown by 16S ribosomal DNA clonal sequence analysis (21). Some members of this phylum, which is related to the known acetate-oxidizing syntrophs, may contribute to the syntrophic acetate oxidation in the chemostat at the low dilution rate.

The results described above, combined with previous findings (8, 21) demonstrate that the dilution rate could cause a shift in the primary pathway of acetate conversion to methane in acetate-fed chemostats. At the low dilution rate, the acetate-oxidizing syntrophs, associated with hydrogenotrophic methanogens, could metabolically overcome the aceticlastic methanogens and play a primary role in the conversion of acetate to methane. Recently, Nüsslein et al. reported that a large proportion of methanogenesis in lake sediment occurs by syntrophic acetate oxidation rather than by aceticlastic cleavage of acetate (15). Most natural environments fulfill the conditions of low dilution rate and low acetate concentration that were present in our chemostat. Syntrophic acetate oxidation might be a common mechanism in natural methanogenic environments.

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REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Boone, D. R., W. B. Whitman, and Y. Koga. 2001. Order III. *Methanosarcinales* ord. nov., p. 268–294. In D. R. Boone and R. W. Castenholz (ed.), *Bergey's manual of systematic bacteriology*, 2nd ed., vol. 1. The *Archaea* and the deeply branching and phototrophic *Bacteria*. Springer-Verlag, New York, N.Y.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783–791.
- Ferry, J. G. 1993. Fermentation of acetate, p. 304–334. In J. G. Ferry (ed.), *Methanogenesis—ecology, physiology, biochemistry & genetics*. Chapman & Hall, New York, N.Y.
- Griffiths, R. I., A. S. Whiteley, A. G. O'Donnell, and M. J. Bailey. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl. Environ. Microbiol.* **66**:5488–5491.
- Hales, B. A., C. Edwards, D. A. Ritchie, G. Hall, R. W. Pickup, and J. R. Saunders. 1996. Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl. Environ. Microbiol.* **62**:668–675.
- Hattori, S., Y. Kamagata, S. Hanada, and H. Shoun. 2000. *Thermoacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. *Int. J. Syst. Evol. Microbiol.* **50**:1601–1609.
- Kida, K., T. Shigematsu, J. Kijima, M. Numaguchi, Y. Mochinaga, N. Abe, and S. Morimura. 2001. Influence of Ni^{2+} and Co^{2+} on methanogenic activity and the amounts of coenzymes involved in methanogenesis. *J. Biosci. Bioeng.* **91**:590–595.
- Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**:1244–1245.
- Lee, M. J., and S. H. Zinder. 1988. Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on $\text{H}_2\text{-CO}_2$. *Appl. Environ. Microbiol.* **54**:124–129.
- Lovley, D. R., and M. J. Klug. 1982. Intermediary metabolism of organic matter in the sediments of a eutrophic lake. *Appl. Environ. Microbiol.* **43**:552–560.
- Lueders, T., K.-J. Chin, R. Conrad, and M. Friedrich. 2001. Molecular analyses of methyl-coenzyme M reductase α -subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ. Microbiol.* **3**:194–204.
- Mackie, R. I., and M. P. Bryant. 1981. Metabolic activity of fatty acid-oxidizing bacteria and the contribution of acetate, propionate, butyrate, and CO_2 to methanogenesis in cattle waste at 40 and 60°C. *Appl. Environ. Microbiol.* **41**:1363–1373.
- Mountfort, D. O., and R. A. Asher. 1978. Changes in proportions of acetate and carbon dioxide used as methane precursors during the anaerobic digestion of bovine waste. *Appl. Environ. Microbiol.* **35**:648–654.
- Nüsslein, B., K.-J. Chin, W. Eckert, and R. Conrad. 2001. Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical Lake Kinneret (Israel). *Environ. Microbiol.* **3**:460–470.
- Petersen, S. P., and B. K. Ahring. 1991. Acetate oxidation in a thermophilic anaerobic sewage-sludge digester: the importance of non-aceticlastic methanogenesis from acetate. *FEMS Microbiol. Ecol.* **86**:149–158.
- Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
- Schnürer, A., F. P. Houwen, and B. H. Svensson. 1994. Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration. *Arch. Microbiol.* **162**:70–74.
- Schnürer, A., B. Schink, and B. H. Svensson. 1996. *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. *Int. J. Syst. Bacteriol.* **46**:1145–1152.
- Schnürer, A., B. H. Svensson, and B. Schink. 1997. Enzyme activities and energetics of acetate metabolism by the mesophilic syntrophically acetate-oxidizing anaerobe *Clostridium ultunense*. *FEMS Microbiol. Lett.* **154**:331–336.
- Shigematsu, T., Y. Tang, H. Kawaguchi, K. Ninomiya, J. Kijima, T. Kobayashi, S. Morimura, and K. Kida. 2003. Effect of dilution rate on structure of a mesophilic acetate-degrading methanogenic community during continuous cultivation. *J. Biosci. Bioeng.* **96**:547–558.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **24**:4876–4882.
- Zinder, S. H. 1993. Physiological ecology of methanogens, p. 128–206. In J. G. Ferry (ed.), *Methanogenesis—ecology, physiology, biochemistry & genetics*. Chapman & Hall, New York, N.Y.
- Zinder, S. H., and M. Koch. 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Arch. Microbiol.* **138**:263–272.