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Functional patterns and temperature response of cellulose-fermenting microbial cultures containing different methanogenic communities

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Abstract The effect of microbial composition on the methanogenic degradation of cellulose was studied using two lines of anaerobic cellulose-fermenting methanogenic microbial cultures at two different temperatures: that at 15 °C being dominated by *Methanosaeta* and that at 30 °C by *Methanosarcina*. In both cultures, CH_4 production and acetate consumption were completely inhibited by either 2-bromoethanesulfonate or chloroform, whereas H_2 consumption was only inhibited by chloroform, suggesting that homoacetogens utilized H_2 concomitantly with methanogens. Hydrogen was the intermediate that was consumed first, while acetate continued to accumulate. At 15 °C, acetoclastic methanogenesis smoothly followed H_2-dependent CH_4 production. Fluorescence in situ hybridization showed that populations of *Methanosaeta* steadily increased with time from 5 to 25% of total cell counts. At 30 °C, two phases of CH_4 production were obtained, with acetate consumed after the abrupt increase of *Methanosarcina* from 0 to 45% of total cell counts. Whereas populations of *Methanosaeta* were able to adapt after transfer from 15 to 30 °C, those of *Methanosarcina* were not, irrespective of during which phase the cultures were transferred from 30 °C to 15 °C. Our results thus show that the community structure of methanogens indeed affects the function of a cellulose-fermenting community with respect to temperature response.

Introduction

Microbial communities can be extremely complex. One gram of soil, for example, was found to contain more than 4,000 different microbial genomes (Torsvik et al. 1990). The question arises which of these organisms are necessary for which function in the ecosystem? This question also pertains to simpler microbial communities, such as those in anaerobic digestion. Recently, Fernandez et al. (1999) showed that a stably performing anaerobic bioreactor exhibited tremendous variation in the composition of its microbial community. The changes were more dynamic in the bacterial than the archaeal community. More recently (Fernandez et al. 2000), these scientists showed that a more flexible community structure correlated with stable community function in methanogenic bioreactors perturbed by a pulse of glucose. They also showed that processing of glucose by a bacterial community with parallel fermentation pathways results in higher stability than by one that processed the substrate sequentially (Hashsham et al. 2000). However, the possible effect of the archaeal community on the anaerobic bioprocess remained unclear.

Recently, we have established two functionally stable microbial communities that degrade cellulose anaerobically to CH_4 and CO_2 (Chin et al. 1999). These communities originated from anoxic rice-field soil and were maintained in two separate culture lines at either 15 or 30 °C. The acetoclastic methanogenic archaeal community of the 15 °C culture line was dominated by phylogenetically closely related *Methanosaeta* species, whereas the 30 °C culture line was dominated by phylogenetically closely related *Methanosarcina* species. Despite the different methanogenic communities, both culture lines had the same temperature optimum and apparent activation energy, i.e., they seemed to be functionally similar (Chin et al. 1999).

In the present report, we studied the two cellulose-degrading methanogenic culture lines in more detail by determining the production of fermentation intermediates and the effect of methanogenic inhibitors. The change in the composition of the archaeal community was also followed using fluorescence in situ hybridization (FISH). In particular, the effect of a shift in the incubation temperature was examined. We found that the 15 °C- and 30 °C culture lines behaved differently, indicating that the composition of the acetoclastic archaeal community indeed affects the functional performance at the two different temperatures.

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Materials and methods

Methanogenic microbial community

The cellulose-degrading methanogenic microbial community consisted of two lines of enrichment cultures that were kept in triplicate at 15 and 30 °C. These cultures had been started by inoculating rice-field soil into anaerobic mineral medium supplemented with cellulose as sole energy substrate (Chin et al. 1999). The cultures were regularly transferred into fresh media. Usually, after incubation for 25–30 days at 30 °C and 110–120 days at 15 °C, 5-ml suspensions of the culture were transferred to serum bottles (120 ml) containing 45 ml mineral medium and a 2-ml suspension of amorphous cellulose (2.5 gl^{-1}) under an atmosphere of 20% CO_2 and 80% N_2 (Chin et al. 1999). After five transfers, the production of CH_4 became reproducible, and the cultures exhibited a relative stable archaeal community (Chin et al. 1999). To study the physiological functioning of the cultures, we used one of the three replicates after the 27th transfer at 30 °C and the 14th transfer at 15 °C as inoculum. Five-ml suspensions were inoculated into nine replicates of fresh anaerobic cellulose medium (the same as described above) and incubated without agitation in the dark. Inhibition studies using 10 mM 2-bromoethanesulfonate (BES) or 50 µM $CHCl_3$ were done with three replicate cultures in the 22nd and 10th transfer of the 30 °C and 15 °C culture line, respectively. Chloroform and BES are used as inhibitors of methanogenesis (Lovley and Klug 1982; Oremland and Capone 1988; Chin and Conrad 1995). Chloroform also inhibits homoacetogenesis from H_2 plus CO_2 (Conrad and Klose 2000; Scholten et al. 2000). For studying the reaction of the cultures towards a shift in incubation temperature, we used each of the three replicates after the 43rd transfer at 30 °C and the 18th transfer at 15 °C as inoculum to generate three lines, three replicates each, that were shifted from either 30 °C to 15 °C or from 15 °C to 30 °C, respectively.

Physiological functioning of the cultures

During the incubation, gas samples (0.1 ml) were repeatedly taken from the gas phase of the bottles with gas-tight syringes and analyzed immediately by gas chromatography (Conrad et al. 1987). Methane was analyzed in a gas chromatograph (GC) with a flame ionization detector (FID) after separation on a Porapak Q column with synthetic air (20% O_2, 80% N_2; Messer Griesheim, Siegen, Germany) as carrier gas. Hydrogen was analyzed in a GC with a reductive gas detector (RGD2; Trace Analytical, Menlo Park, Calif., USA) after separation on a molecular sieve column with synthetic air as carrier gas. Liquid samples (0.5 ml) were also taken frequently from the bottles, filtered through 0.2-µm RC-Membrane filters (Minisart, Regenerated Cellulose 15, Sartorius, Göttingen, Germany), and stored frozen at –20 °C till analysis (Roy et al. 1997). Fatty acids in the liquid samples were analyzed by high pressure liquid chromatography (HPLC) using a refractive index (RI) detector (Sykam, Gauting, Germany) with a detection limit of 5 µM of the individual fatty acids (Krumböck and Conrad 1991).

Fluorescence in situ hybridization

The community dynamics and effects of temperature shift of the archaeal community were analyzed by FISH. Liquid samples (1 ml) were repeatedly taken from the cultures, the microorganisms were fixed with paraformaldehyde, and the samples hybridized with fluorescently-labeled 16S rRNA probes according to the standard protocol described by Amann et al. (1990, 1996). Before hybridization, the fixed cells were usually stored for up to 1 day at –20 °C without loss of detectability. The probes were labeled with fluorescein, Texas red, and CY5 (MWG-Biotech, Ebersberg, Germany). The probes Arc915 and Arc344 were used for detecting the archaea in general; probe MS821 was used for detection of the genus *Methanosarcina*; and probe MX825 was used for detection of *Methanosaeta* (Raskin et al. 1994; Amann et al. 1995). For estimation of the relative abundance of the different groups of methanogens, double hybridization with a specific probe together with the two general archaeal probes was done. The total population of microbial cells was counter-stained with 4',6-diamidino-2-phenylindole (DAPI), washed with distilled water, and embedded with Citiflour (Citifluor, Canterbury, UK). The stained microorganisms were visualized using a confocal laser scanning microscope (Leica TCS-NT, Leica, Heidelberg, Germany). For enumeration, ten microscopic views were counted for each sample, resulting in about 200–1,200 cells stained with the general archaeal probe (Chin et al. 1999). Relative abundance was evaluated as the percentage of cells that hybridized with one of the specific or general archaeal probes relative to those stained with DAPI.

Results

Culture line at 30 °C

The initial time course of accumulation of CH_4, H_2 and acetate in the cellulose-degrading culture at 30 °C was similar to that reported by Chin et al. (1999). In the present experiments, the incubation time was extended until the accumulated acetate was completely depleted (Fig. 1). Under these conditions, propionate was also detected as an intermediate; it accumulated transiently and was almost depleted by the end of the incubation. The prolonged incubation revealed that CH_4 production occurred in two phases. Methane started to increase after 1 day, accelerated after 5 days, reached a plateau after 10 days, and again increased after 40 days of incubation, reaching a final partial pressure of about 10 kPa (Fig. 1A). Hydrogen transiently accumulated to partial pressures of 120 Pa during the first phase of CH_4 accumulation, but then decreased to levels below 1 Pa. It slightly increased again to constant values around 2 Pa when propionate consumption started (Fig. 1B, D). Acetate began to accumulate from the beginning of incubation, reached a plateau at about 9 mM approximately in parallel with CH_4, and decreased again when CH_4 entered the second phase of accumulation (Fig. 1C). The same accumulation pattern was observed with propionate, but its consumption started later than that of acetate (Fig. 1D). The pH of the cultures was constant, between 6.8 and 7.2, throughout the experiment. In some samples, formate, lactate, butyrate, iso-butyrate, valerate, caproate and iso-propanol were also detected, but the concentrations of these compounds were usually low (20–500 µM) and they did not exhibit a clear accumulation pattern.

We also quantified the archaeal community by FISH relative to the total microbial cells counted by DAPI (Fig. 1E). The cultures were assayed with general archaeal probes (Arc915 plus Arc344) and a probe specific for *Methanosarcina* (MS821), the difference reflecting non-*Methanosarcina* cells. We were unable to detect any of the typical *Methanosaeta* filaments in the 30 °C cultures. Acetoclastic methanogens, represented by *Methanosarcina* cells, were not detectable until 23 days of incubation, but increased thereafter and reached a population density of about 30% of total microbial cells

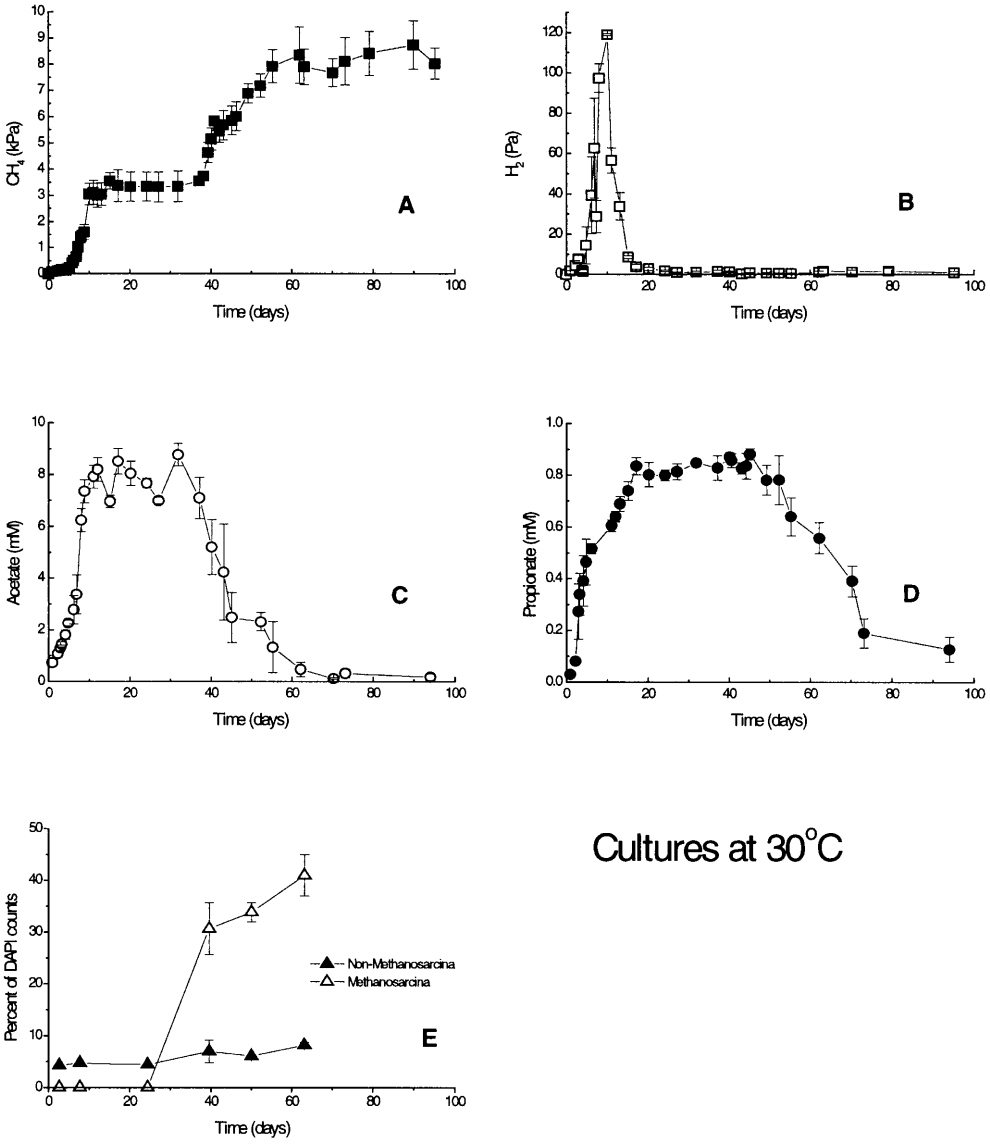

Fig. 1A–E Temporal change during anaerobic cellulose degradation of **A** CH_4 partial pressure, **B** H_2 partial pressure, **C** acetate concentration, **D** propionate concentration, and **E** abundance of archaeal cells in an anaerobic culture line at 30 °C; means±SE of *n*=9

(Fig. 1E) at the time when CH_4 production entered the second phase (Fig. 1A) and acetate started to decrease (Fig. 1C). In the end, the *Methanosarcina* cells made up about 45% of the total microbial cells. Other archaeal cells, although detectable right from the beginning of incubation, increased only slightly, from 4% to 8% of total cell counts during the course of the experiment (Fig. 1E).

Inhibition experiments were carried out separately. The uninhibited control again showed the typical two phases of CH_4 production and the transient accumulation of H_2, acetate and propionate was quite similar to that in Fig. 1 (data not shown). Addition of either BES or $CHCl_3$ resulted in the complete inhibition of CH_4 production. In addition, the inhibitors resulted in the accumulation of intermediates, which either reached higher values, lasted longer, or reached a constant value that did not decrease later on when compared to the control (Table 1). Noteworthy was the permanent accumulation of H_2 and formate in the presence of $CHCl_3$ but not of BES, indicating that $CHCl_3$, but not BES, inhibited consumption of H_2 and formate. Acetate and propionate, by contrast, were no longer consumed in the presence of both BES and $CHCl_3$.

Culture line at 15 °C

The initial time course of accumulation of CH_4, H_2 and acetate in the cellulose-degrading culture at 15 °C was similar to that reported by Chin et al. (1999). In the present experiments the incubation time was extended until the accumulated acetate was again depleted (Fig. 2). Propionate was also detected but it was not depleted until the end of the experiment. Methane started to increase after about 25 days of incubation (Fig. 2A). Some 17 days later, H_2 began to increase; it reached a maximum at around 2,000 Pa and decreased to constant and low partial pressures of <10 Pa after about 70 days of incubation

Table 1 Effect of methanogenic inhibitors on the accumulation pattern of intermediates

Accumulated intermediate	Control		BES		$CHCl_3$	
	Maximum	Duration (h)	Maximum	Duration (h)	Maximum	Duration (h)
30 °C						
H_2 (kPa)	1	250	1.5	700	4.8	Permanent
Formate (mM)	<0.05	0	0.4	50	0.75	Permanent
Acetate (mM)	5	900	7	Permanent	3	Permanent
Propionate(mM)	0.6	1600	0.55	Permanent	0.6	Permanent
15 °C						
H_2 (kPa)	2.8	700	0.8	300	2.1	Permanent
Formate (mM)	0.4	2500	0.3	120	0.3	Permanent
Acetate (mM)	5	2000	5	Permanent	5	Permanent
Propionate (mM)	0.5	Permanent	1.5	Permanent	1	Permanent

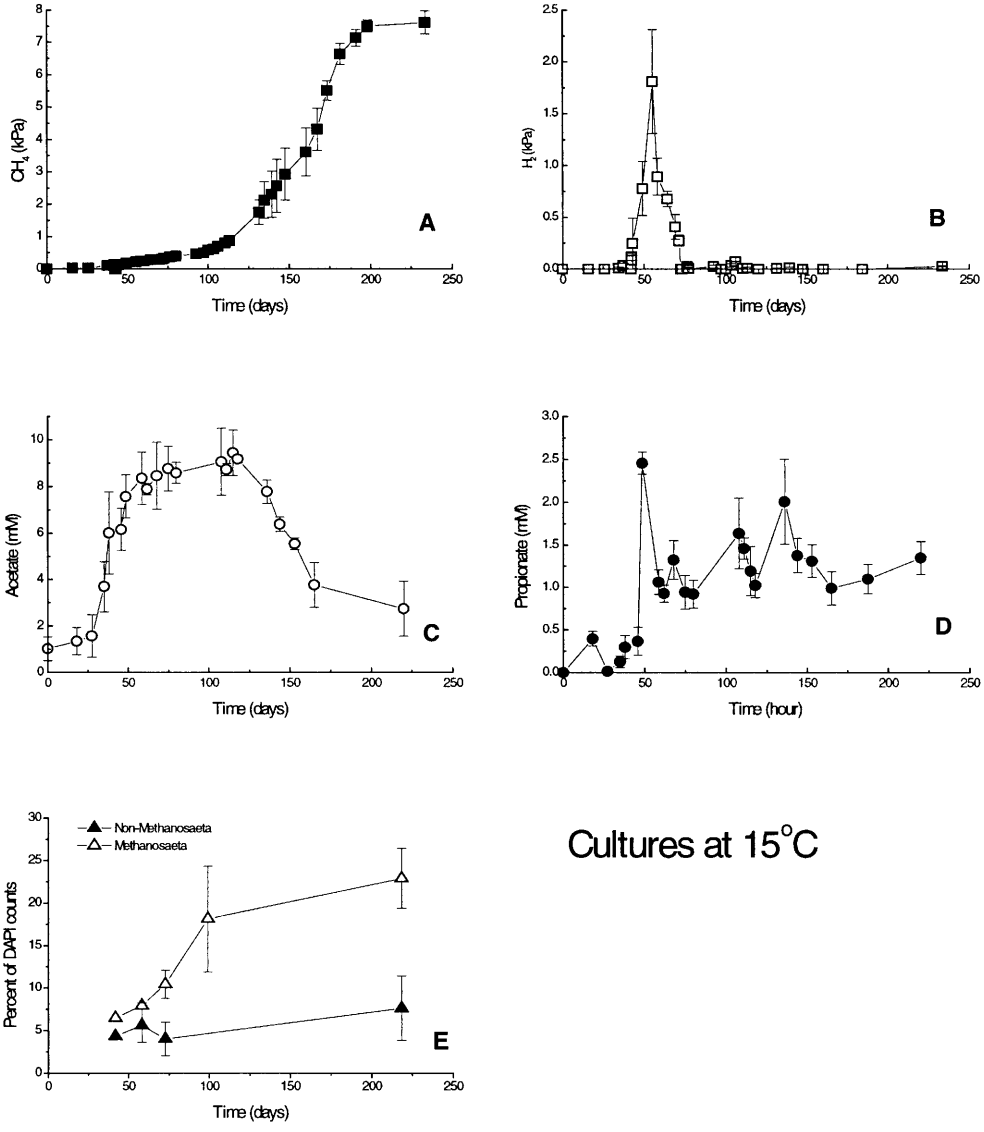

Fig. 2A–E Temporal change during anaerobic cellulose degradation of **A** CH_4 partial pressure, **B** H_2 partial pressure, **C** acetate concentration, **D** propionate concentration, and **E** abundance of archaeal cells in an anaerobic culture line at 15 °C; means±SE of n=9

(Fig. 2B). Acetate and propionate accumulated during this phase to about 9 mM and 1.5 mM, respectively (Fig. 2C, D). Production of CH_4 accelerated after about 125 days of incubation, concomitant with the decrease of acetate, and reached a final partial pressure of about 8 kPa (Fig. 2A, C). There was a slight shoulder in the CH_4 production curve between the end of H_2 consumption and the beginning of acetate consumption, but an interruption of CH_4 production between an initial and a second phase as in the 30 °C cultures was not observed.

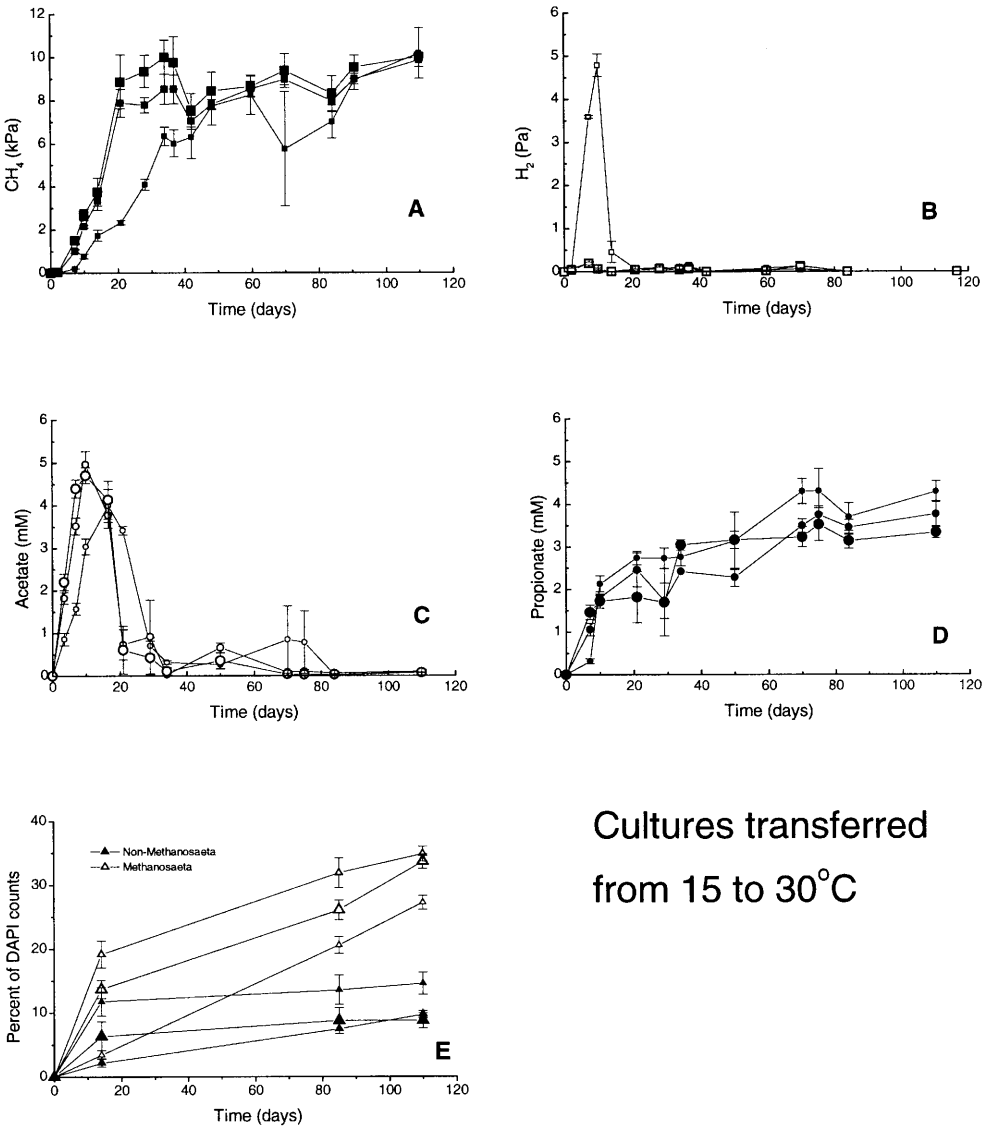

Fig. 3A–E Temporal change during anaerobic cellulose degradation of **A** CH_4 partial pressure, **B** H_2 partial pressure, **C** acetate concentration, **D** propionate concentration, and **E** abundance of archaeal cells in three different anaerobic culture lines transferred from 15 to 30 °C; means±SE of *n*=3

The 15 °C cultures were assayed with general archaeal probes (Arc915 plus Arc344) and a probe specific for *Methanosaeta* (MX825), the difference reflecting non-*Methanosaeta* cells (Fig. 2E). In contrast to the 30 °C cultures, acetoclastic methanogens in the form of *Methanosaeta* cells were found from the beginning of CH_4 production and steadily increased during the course of the incubation from about 6% to 20% of total microbial cells. Archaeal cells other than *Methanosaeta* were also detected throughout the incubation and slightly increased from about 4% to 7% of total microbial cells by the end of the incubation. We cannot exclude that the fraction of non-*Methanosaeta* archaeal cells may have contained some *Methanosarcina* cells.

Inhibition experiments with BES and $CHCl_3$ showed inhibition patterns similar to those at 30 °C (Table 1). Again, $CHCl_3$ but not BES inhibited the consumption of transiently accumulated H_2 and formate. BES even decreased and shortened the accumulation of H_2 and formate, indicating that these intermediates were then converted to products other than CH_4, possibly acetate.

Temperature shift

The response of the 15 °C- and 30 °C culture lines following transfer to the other temperature was examined. The temperature of the 15 °C culture lines was shifted to 30 °C after 170 days of growth, concomitant with the transfer to new medium (Fig. 3). The temperature of the 30 °C culture lines was shifted to 15 °C after two different times of growth. The first batch was shifted concomitant with the transfer to new medium after about 8 days, i.e., during the first phase of CH_4 production (Fig. 4), and the second batch after about 56 days, i.e., during the second phase of CH_4 production (Fig. 5).

The shift of the 15 °C cultures to 30 °C resulted in accelerated CH_4 (Fig. 3A) production with only a brief phase of transient accumulation of H_2 (Fig. 3B) and acetate (Fig. 3C). Both methanogenic substrates were apparently utilized simultaneously. Propionate, on the other hand, accumulated to about 3.5 mM and was not consumed during the course of the experiment (Fig. 3D).

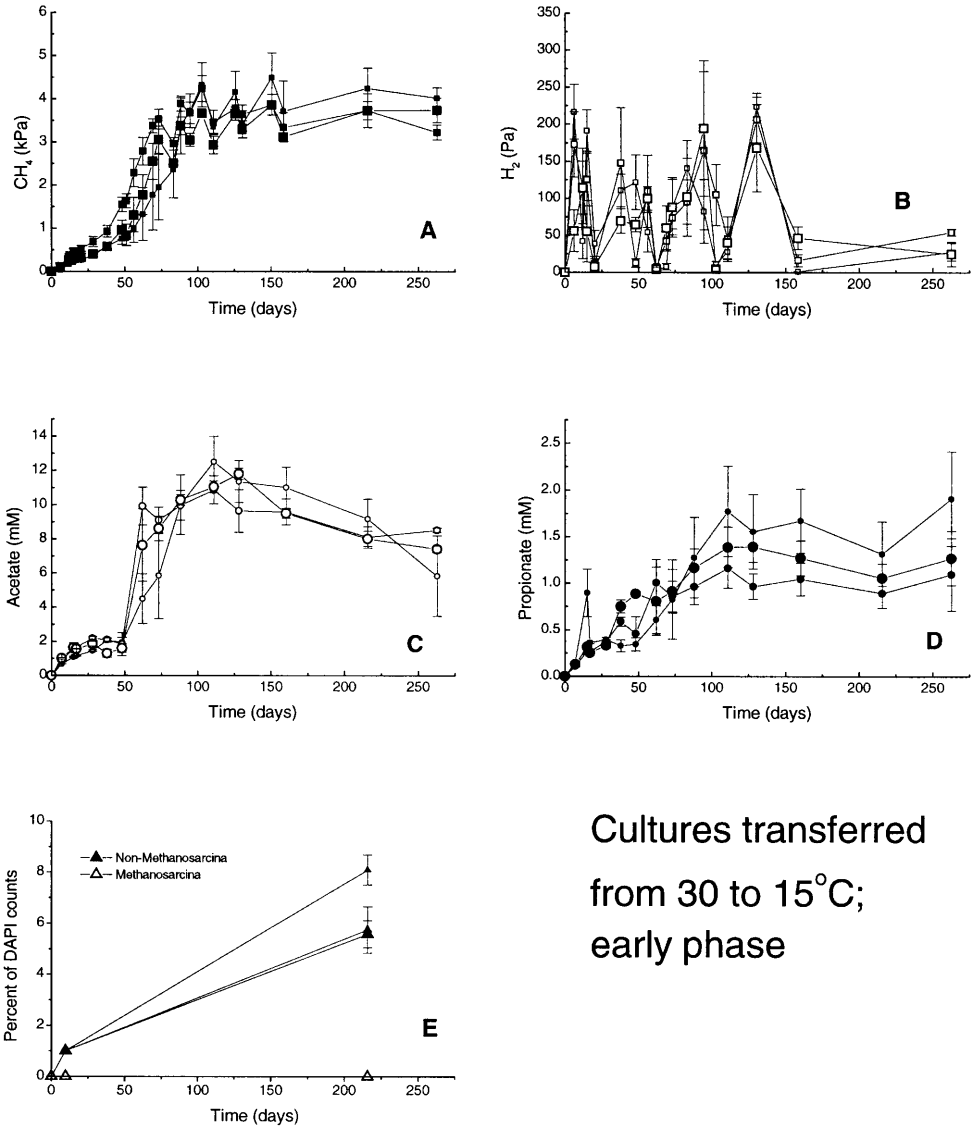

Fig. 4A–E Temporal change during anaerobic cellulose degradation of **A** CH_4 partial pressure, **B** H_2 partial pressure, **C** acetate concentration, **D** propionate concentration, and **E** abundance of archaeal cells in three different anaerobic culture lines transferred from the early phase at 30 to 15 °C; means±SE of *n*=3

The numbers of *Metanosaeta* cells, as determined by FISH, continuously increased during the experiment to about 25–35% of total cell counts, those of archaeal non-*Methanosaeta* cells increased only to 8–14% of total cell counts (Fig. 3E). The presence of some *Methanosarcina* cells among the latter group cannot be excluded.

By contrast, the shift of the 30 °C cultures to 15 °C resulted in a reduced production of CH_4. Irrespective of whether the inoculum was taken during the first (Fig. 4A) or second (Fig. 5A) phase of CH_4 production, CH_4 accumulated to only a fraction of what was obtained in this type of cultures at 30 °C (Fig. 1A) or in the 15 °C cultures at either 15 °C (Fig. 2A) or 30 °C (Fig. 3A). The cultures obtained with the inoculum taken from the second phase of CH_4 production also showed a relatively large variability, so that one of the triplicates was more active than the others (Fig. 5). Furthermore, these cultures produced relatively smaller amounts of acetate and propionate compared to cultures that were inoculated with material from the first phase of CH_4 production (Fig. 5), indicating a reduction of capacity to ferment cellulose. In both cultures, acetate accumulated without any net consumption during the course of the experiments, indicating that, in particular, acetoclastic methanogenesis was missing. This conclusion is consistent with our inability to detect any *Methanosarcina* cells by FISH in either of the cultures. In addition, archaeal cells were only detectable in the cultures that were inoculated with material from the first phase of CH_4 production, but only at a low percentage (Fig. 4E). The cultures from the latter inoculum did not contain any detectable archaeal cells. Obviously, the *Methanosarcina* cells that were originally present in the inoculum must have been inactivated rapidly due to the decreased temperature, so that staining by FISH was no longer possible.

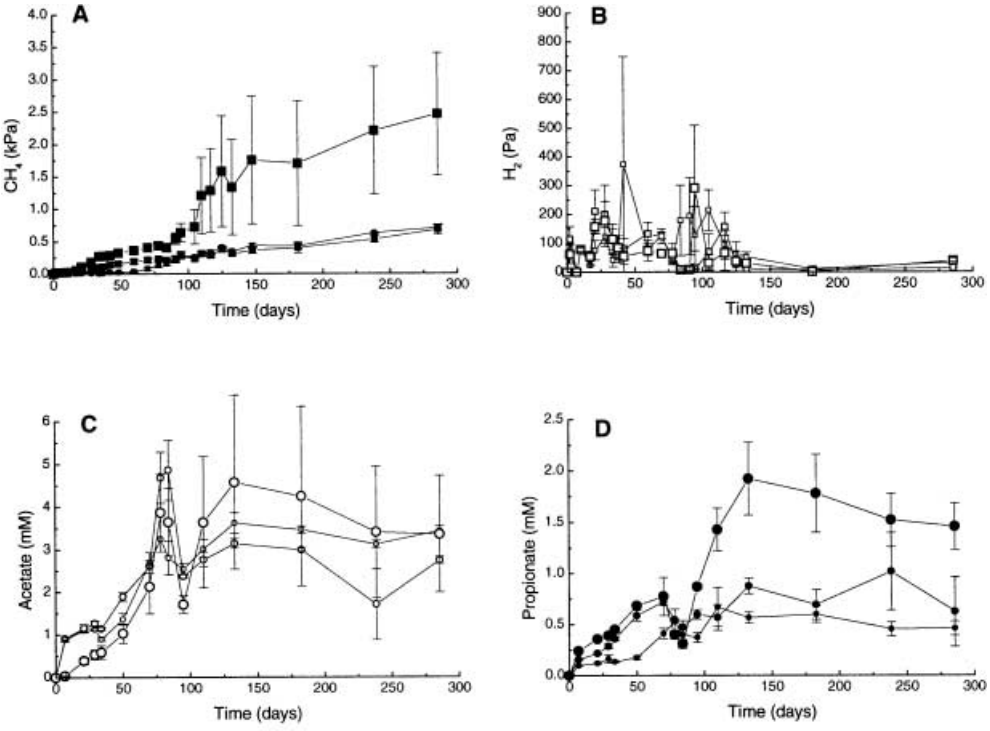

Fig. 5A–D Temporal change during anaerobic cellulose degradation of **A** CH_4 partial pressure, **B** H_2 partial pressure, **C** acetate concentration, **D** propionate concentration in three different anaerobic culture lines transferred from the late phase at 30 to 15 °C; means±SE of *n*=3

Discussion

Cultures transferred from 30 to 15°C; late phase

The cellulose-degrading cultures exhibited, despite their different incubation temperatures and their different archaeal communities, a similar functional pattern. At both 15 °C and 30 °C, cellulose was degraded to CH_4 with the transient accumulation of propionate, H_2 (and formate), and acetate as intermediates. Intermediates that serve as direct methanogenic precursors, i.e., H_2 (formate) and acetate, were degraded later. However, propionate was only degraded at 30 °C but not at 15 °C. The methanogenic inhibitor BES inhibited CH_4 production at both temperatures completely and acetate was no longer degraded. In addition, chloroform inhibited the further utilization of H_2 and formate. We thus conclude that both the 15 °C and 30 °C culture lines contained microorganisms able to utilize H_2 and formate in the presence of BES but not chloroform, presumably homoacetogenic bacteria that form acetate from H_2 plus CO_2 (Conrad and Klose 2000; Scholten et al. 2000).

The temporal pattern of CH_4 production at the two different temperatures was also similar, with methanogenic H_2 utilization operating first and acetate utilization second. The only difference was that this sequential operation occurred in two well-separated phases at 30 °C, while it merged into only one obvious phase at 15 °C. This difference was probably due to the different activation and/or growth of *Methanosarcina* and *Methanosaeta* populations dominating the 30 °C and 15 °C cultures, respectively.

Although the general functional patterns of cellulose degradation were similar, the 15 °C and 30 °C culture lines greatly differed with respect to their reaction to a shift in the incubation temperature. The *Methanosarcina*-dominated cultures reacted sensitively when the temperature was changed from 30 °C to 15 °C, and active *Methanosarcina* could no longer be detected by FISH. It is known that the detection of microbial cells by FISH depends on a sufficient number of ribosomes, which will be too low if the cells are dormant, starved, or growing at a low rate (Amann et al. 1995; Oda et al. 2000; Ramos et al. 2000). Obviously, the *Methanosarcina* populations in the 30 °C culture lines were sensitive to low temperature. In contrast, the *Methanosaeta* populations in the 15 °C culture lines survived the shift to a higher temperature and immediately continued growth. The non-*Methanosaeta* populations also proliferated but reached only a relatively low abundance. The different reactions of the dominant acetoclastic methanogenic populations upon temperature shifting was reflected in the functional patterns, i.e., accumulation and consumption of acetate. A review of pure culture studies showed no difference in the temperatures range of *Methanosarcina* vs *Methanosaeta* species, which were all in the mesophilic or thermophilic range (Boone et al. 1993). Our study showed, however, that *Methanosaeta*-dominated cultures tolerated an up-shift in temperature, while

Methanosarcina-dominated cultures did not tolerate a down-shift. Hence, a decrease in temperature may be detrimental for the performance of mesophilic methanogenic bioreactors.

Our study showed that functionally similar but structurally different methanogenic archaeal communities can have a decisive effect on the reaction of a methanogenic system to temperature shifts. However, the composition of the bacterial community can also have strong effects (Hashsham et al. 2000). In our study, for example, the composition of the bacterial community may have affected the metabolism of propionate, as the accumulated propionate was only utilized at 30 °C. We speculate that the low temperatures selected against propionate-utilizing microorganisms. This assumption is plausible, since the standard Gibbs free energy of syntrophic propionate degradation becomes increasingly endergonic with decreasing temperature. Consistent with the thermodynamics, it has been found that carbon flow via propionate decreased following a temperature shift from 30 °C to 15 °C in methanogenic rice-field soil (Chin and Conrad 1995).

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