Physiological and molecular characterizations of the interactions in two cellulose-to-methane cocultures

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Abstract

Background: The interspecies interactions in a biomethanation community play a vital role in substrate degradation and methane (CH₄) formation. However, the physiological and molecular mechanisms of interaction among the microbial members of this community remain poorly understood due to the lack of an experimentally tractable model system. In this study, we successfully established two coculture models combining the cellulose-degrading bacterium *Clostridium cellulovorans* 743B with *Methanosarcina barkeri* Fusaro or *Methanosarcina mazei* Gö1 for the direct conversion of cellulose to CH₄.

Results: Physiological characterizations of these models revealed that the methanogens in both cocultures were able to efficiently utilize the products produced by *C. cellulovorans* during cellulose degradation. In particular, the simultaneous utilization of hydrogen, formate, and acetate for methanogenesis was observed in the *C. cellulovorans*–*M. barkeri* cocultures, whereas monocultures of *M. barkeri* were unable to grow with formate alone. Enhanced cellulose degradation was observed in both cocultures, and the CH₄ yield of the *C. cellulovorans*–*M. barkeri* cocultures (0.87 ± 0.02 mol CH₄/mol glucose equivalent) was among the highest compared to other coculture studies. A metabolic shift in the fermentation pattern of *C. cellulovorans* was observed in both cocultures. The expression levels of genes in key pathways that are important to the regulation and metabolism of the interactions in cocultures were examined by reverse transcription-quantitative PCR, and the expression profiles largely matched the physiological observations.

Conclusions: The physiological and molecular characteristics of the interactions of two CH₄-producing cocultures are reported. Coculturing *C. cellulovorans* with *M. barkeri* or *M. mazei* not only enabled direct conversion of cellulose to CH₄, but also stabilized pH for *C. cellulovorans*, resulting in a metabolic shift and enhanced cellulose degradation. This study deepens our understanding of interspecies interactions for CH₄ production from cellulose, providing useful insights for assembling consortia as inocula for industrial biomethanation processes.

Keywords: Methanogenesis, Dark fermentation, Coculture, Cellulose degradation, Transcriptome, Metabolism

Background

Biomethanation, a natural biological process by which organic materials are transformed into biogas, can be deployed for waste treatment and sustainable energy production [1, 2]. This process has been widely applied in municipal sewage treatment to not only effectively reduce the volume and odor of volatile solids, but also produce methane (CH₄) as an energy resource to power treatment facilities [3]. As the worldwide demand for renewable energy increases, biomethanation of organic materials has an important role to play in our energy future [4].

The biomethanation process in nature relies on the microbial interactions between three main metabolic groups of anaerobes: fermentative, acetogenic, and methanogenic microorganisms [5–7]. The first two groups decompose complex organic matters to acetate, hydrogen
(H₂), and carbon dioxide (CO₂), which are the key precursors for methanogenesis. Methanogens further convert these metabolites to CH₄ by two major routes: the acetoclastic and CO₂ reduction pathways [8]. Although methanogens are obligately dependent on the first two metabolic groups to supply substrates for growth, the two methanogenesis pathways can in turn affect their activities. First, H₂ production by some bacteria is thermodynamically unfavorable; therefore, their growth is contingent on the CO₂-reducing methanogens to maintain a low H₂ partial pressure [9]. For example, the maintenance of a very low concentration of H₂ in the ecosystem by the methanogens is essential for the catabolism of fatty acids by the obligate proton-reducing acetogenic bacteria Desulfovibrio vulgaris [10, 11]. Second, the consumption of acetate by acetate-utilizing methanogens can help to maintain a pH close to neutral to support optimal metabolic activities of other members [2]. These illustrated interdependent relationships form the basis of many interactions that occur in a biomethanation community.

Because cellulosic materials are commonly found in nature, methanogens often exist concomitantly with cellulose-fermenting bacteria in anaerobic habitats, such as sediments, sewage digesters, and landfills [12–14]. Cellulose-fermenting bacteria hydrolyze the insoluble cellulose into end products—such as organic acids, CO₂, and H₂—which become carbon and energy sources for other members, including methanogens, within the microbial community. Because of this substrate dependency, the interactions between the cellulosytic bacteria and methanogens play a crucial role in shaping a biomethanation community. In order to gain insights into the metabolic functions of the cellulosytic-methanogenic communities, efforts have been made to study the interactions between cellulose-fermenting bacteria and H₂/formate/acetate-consuming methanogens in artificially constructed cultures. For example, Laube and Martin [15] studied cocultures of Acetivibrio cellulolyticus—Methanosarcina barkeri and M. barkeri—Desulfovibrio sp., as well as a triculture integrating A. cellulolyticus, M. barkeri, and Desulfovibrio sp. Their results showed that the methanogen was able to utilize the H₂ and acetate produced by the cellulose-fermenting bacteria for CH₄ production, resulting in improved CH₄ production and a faster fermentation rate in the triculture. Nakashimada et al. [16] investigated cocultures of the anaerobic fungi Neocallimastix frontalis with a formate- and H₂-utilizing methanogen (Methanobacterium formicicum) or an acetoclastic methanogen (Methanosaeta concilii), as well as a triculture incorporating N. frontalis, M. formicicum, and M. concilii. Their results demonstrated that whereas the coculture of N. frontalis—M. formicicum utilized formate and H₂ and the coculture of N. frontalis—M. concilii utilized acetate for CH₄ production, the triculture of N. frontalis, M. formicicum, and M. concilii was able to use formate, H₂, and acetate for CH₄ production. Robert et al. [17] investigated interspecies H₂ transfer by employing cocultures of fibrolytic bacteria and the H₂-utilizing colonic methanogen Methanobrevibacter smithii and observed that H₂ utilization by the methanogen induced a metabolic shift in the cellulosytic strain. Sasaki et al. [18] incorporated C. clariflavum CL-1 and the hydrogenotrophic methanogen Methanothermobacter thermautotrophicus ∆H under thermophilic conditions. They reported that the cellulose degradation efficiency and cell density of C. clariflavum CL-1 were significantly higher in the coculture than in the monoculture. Bauchop et al. [19] employed a rumen anaerobic fungus with a consortium of rumen methanogens for methanogenesis from cellulose and observed a metabolic shift in the fungus.

Although the aforementioned coculture and triculture studies have all demonstrated that CH₄ can be successfully produced from products of cellulose hydrolysis by various methanogens, the simultaneous utilization of H₂, formate, and acetate by a single methanogen in a coculture has not yet been reported. In addition, the molecular mechanism of the interactions between fermentative cellulose degraders and methanogens is also unclear; however, such an understanding is essential in order to shed light on key cellular regulation and metabolism during coculturing. For instance, gene expression of key pathways related to the metabolic shift in cellulosytic bacteria and the activity of methanogen is of great importance to understand the carbon and electron flows between these two organisms. In this study, we examined whether the metabolic versatile methanogen Methanosarcina barkeri Fusaro—which is potentially capable of utilizing H₂, formate, and acetate for methanogenesis—can form a coculture with the cellulosic-degrading bacterium Clostridium cellulovorans 743B, which is capable of producing all three methanogenesis precursors (H₂, formate, and acetate) as major fermentation metabolites. Meanwhile, Methanosarcina mazei Gö1, which possesses the ability to utilize H₂ and acetate but not formate for methanogenesis, was also employed for coculturing with C. cellulovorans to enable comparison with the C. cellulovorans—M. barkeri cocultures. The genomes of C. cellulovorans, M. barkeri, and M. mazei [20–22] have all been fully sequenced, making the two cocultures genetically tractable in order to understand the molecular mechanisms of the interactions. The physiology of the two coculture models was characterized, and the expression levels of genes in key pathways in cocultures and monocultures were analyzed and compared. Overall, the results of this study provide insights into the interactions between the
cellulolytic bacterium and the methanogens, and a comprehensive understanding of these interactions is crucial for engineering synthetic consortia for large-scale biomethanation processes to produce energy from renewable cellulosic biomass.

Methods

Cultures and growth conditions

The dark fermentative bacterium C. cellulosovorans (ATCC# 35296) was purchased from the American Type Culture Collection (ATCC, VA, USA), whereas the two methanogens—M. barkeri (DSM# 804) and M. mazei (DSM# 3647)—were purchased from the German Collections of Microorganisms and Cell Cultures (DSMZ, Germany). C. cellulosovorans, M. barkeri, and M. mazei were first grown in the respective media recommended by the culture collections to revive the lyophilized cells. Subsequently, active inoculum (5% vol/vol) of C. cellulosovorans was transferred to a defined medium (DCB-1) with 3 g/L of cellulose as previously described [23] with the following modifications: 2 g/L of yeast extract, 10 g/L of sodium chloride, and 9.5 g/L of magnesium sulfate were added, and CO₂/N₂ (20%:80%) filled the headspace. Active inocula (10% vol/vol) of M. barkeri and M. mazei were transferred to a defined high-salt medium [24] with 50 mM of acetate and CO₂/N₂ (20%:80%) in the headspace. After inoculation, the methanogens grown with acetate required about 6 months of acclimation before noticeable growth occurred, but thereafter the cultures could be routinely propagated every 2 weeks.

All monoculture and coculture experiments were carried out in 160-mL serum bottles with 100 mL of the DCB-1 medium as described above and incubated at 35 °C without shaking. Monocultures of C. cellulosovorans were grown with 3 g/L of cellulose, whereas monocultures of M. barkeri and M. mazei were grown with 50 mM of acetate. For coculture experiments, 3 g/L of cellulose was amended as the only substrate, and C. cellulosovorans and M. barkeri were inoculated at a cell ratio of 1:4:1, whereas C. cellulosovorans and M. mazei were inoculated at a cell ratio of 1:7:1. The inoculum for each experiment was obtained from the mid-exponential growth phase of the respective monocultures. To examine whether monocultures of M. barkeri can grow with formate alone, active inoculum of M. barkeri grown with acetate was transferred to the DCB-1 medium and high-salt medium amended with 50 mM of formate. In addition, to evaluate whether the presence of other methanogenesis precursors affected the consumption of formate by M. barkeri, active inoculum of M. barkeri grown with acetate was transferred to the DCB-1 medium amended with all three methanogenesis precursors (10 mM of formate, 3 mM of acetate, and 0.95 mmol of H₂), and to the DCB-1 medium with 3 mM of acetate and 0.95 mmol of H₂ as control. These conditions mimicked the concentrations of the three methanogenesis precursors in the C. cellulosovorans monocultures at mid-exponential growth phase. All experiments were performed in triplicate.

Analytical analyses

The total volume of gas accumulated at each time point was measured. A needle connected to a disposable syringe was inserted through the stopper into the headspace when taking measurement. The volume in the calibrated syringe after plunger displacement was the gas accumulated. The concentrations of H₂ and CH₄ were sampled using a gas-tight syringe (Hamilton, NV, USA) and analyzed using a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with a thermal conductivity detector and a flame ionization detector. The column (30 m × 0.53 mm inner diameter) for H₂ detection was a 5A molecular sieve (Restek, PA, USA) and the column (30 m × 0.53 mm inner diameter) for CH₄ detection was a Rt–QS-BOND column (Restek, PA, USA), both with helium as a carrier gas. The column temperature was 35 °C, the detector temperature was 200 °C, and the injector temperature was 120 °C for both analyses. The CO₂ produced by C. cellulosovorans [25] was not determined since excess CO₂ was provided by filling the headspace of the culture bottles with CO₂/N₂ (20%:80%) and 2.5 g/L of sodium bicarbonate was added as part of the DCB-1 medium. The concentrations of gas (Cᵢ) were converted to total mole (n) of H₂ or CH₄ in each serum bottle using the corresponding Henry’s constant (H) [26] according to the following material balance: 

\[
\text{Gas accumulation} = n = C_G V_G + C_G H V_L
\]

where \( V_G \) and \( V_L \) are the gas and liquid volumes in a serum bottle, respectively. Two mLs of sample were withdrawn from each culture bottle to measure pH, the concentrations of metabolic products and cellulose, and the cell density as described previously [27]. Glucose equivalent is a measure of the amount of reducing sugars present in a sugar product, relative to glucose [28].

Cell morphology analysis

The morphology of cells in the cocultures and monocultures was visualized by scanning electron microscopy (SEM). SEM analysis was performed as previously described [29] with some modifications. Samples were collected from biological triplicate during the mid-exponential growth phase according to the amount of H₂ or CH₄ produced and pooled prior to cell fixation. Cells were fixed for 24 h at 4 °C in 2% vol/vol glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Subsequently, the cells were washed with 0.1, 0.05, and 0.025 M of cacodylate buffers for 15 min each. Samples were then dehydrated through a gradient of ethanol concentrations (50,
70, and 90% vol/vol) for 15 min each, then washed three times with 100% ethanol and acetone for 15 min each. Ethanol–acetone dehydrated samples were critical-point dried in liquid CO₂ with a Bal-Tec CPD 030 critical-point drier (Bal-Tec, Balzers, Liechtenstein). With the use of carbon tape, the samples were stuck on aluminum stubs. The dehydrated samples were observed under a Philips XL30 ESEM FEG environmental SEM (Philips Electron-ics, Netherlands) after being sputter coated with gold palladium using a Bal-Tec SCD 050 sputter coater (Bal-Tec, Balzers, Liechtenstein).

**Cell density analysis**

Genomic DNA extraction and absolute quantification of cell number of the respective organisms in each culture with quantitative PCR (qPCR) were performed as described previously [27]. *C. cellulovorans* was quantified by targeting its cellulase gene (Gene ID: 9607758) with forward primer 5′-ACAGCGCAAGATGCGGTCTCTTA-3′ and reverse primer 5′-GCTGTAGCTCCCCATT-GAGT-3′. *M. Barkeri* by its formate dehydrogenase subunit alpha gene (Gene ID: 3625978) with forward primer 5′-TCGGACC CGGATCTAAACA-3′ and reverse primer 5′-ATTGGTCTGGGTCCCGTTCT-3′, and *M. mazei* by its methyl-coenzyme M reductase gene (Gene ID: 1479582) with forward primer 5′-ATTGGTGCTGGGTCCCGTTCT-3′ and reverse primer 5′-CGACCCTATTTTCCTGAAACCA-3′. One copy of the target gene was found in each respective genome. All primers in this study were designed using Primer Express 3.0 (Applied Biosystems, CA, USA) and their specificity was verified.

**RNA extraction**

Duplicate monocultures or cocultures were prepared for mRNA relative quantification analysis. Cells were collected during the mid-exponential growth phase, and total RNA was extracted using the protocol described previously [30]. The purity and concentration of the total RNA were determined by a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). For all samples, A₃₂₆/Aₘ₈ ratios ranged from 2.0 to 2.1, and concentrations were above 91 ng/µL. The integrity and quality of the total RNA were further assessed on a bioanalyzer 2100 (Agilent, CA, USA) with the Agilent RNA 6000 Pico kit (Agilent, CA, USA) according to the manufacturer’s instructions. Only samples with an RNA integrity number (RIN) above 7.5 were used for downstream analyses.

**Reverse transcription (RT)-qPCR**

To examine the cellular regulation of key pathways of the cocultures and monocultures, 37 genes were selected and analyzed by RT-qPCR using specific primers (Additional file 1: Table S1) for relative mRNA quantification. The genes of *C. cellulovorans* are designated as “Clocel,” *M. Barkeri* as “Mbar A,” and *M. mazei* as “MM.” Total RNA was reverse-transcribed into complementary DNA (cDNA) with random hexamers using the SuperScript III (Invitrogen, CA, USA) reverse transcriptase according to the manufacturer’s instructions. For negative reverse transcriptase controls to verify the absence of genomic DNA, diethylpyrocarbonate-treated water replaced the reverse transcriptase. Amplification of the synthesized cDNA (two technical replicates per biological replicate) and negative controls was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems, CA, USA) using the PowerUp™ SYBR Green Master Mix (Applied Biosystems, CA, USA) according to the manufacturer’s instructions and default thermal-cycling conditions.

Comparative threshold (Ct) differences between cocultures and monocultures were calculated using averages of quadruplicate samples. The fold difference for each target gene was calculated using the 2⁻∆∆Ct method [31] (reported as ratio of coculture/monoculture). Because peptidyl-prolyl isomerase (cyclophilin) is considered a stable housekeeping gene [32, 33], and our previous experiments with *C. cellulovorans* [30] validated the expression of this gene was unaffected by experimental treatment, peptidyl-prolyl isomerase was used as an internal control gene for normalization for *C. cellulovorans*. On the other hand, the glyceraldehyde dehydrogenase (gap) gene of *M. Barkeri* and *M. mazei* [34] was used as an internal control gene for normalization. Specific primers for the three internal control genes are listed in Additional file 1: Table S2. The statistical significance of the expression ratio of each gene between two conditions (cocultures versus monocultures) was analyzed using the Student’s t test. The standard deviation of the fold changes across replicates of each gene was on average equal to an absolute value of 0.6 fold. An absolute value of the fold change ≥1.2 (twice the average standard deviation across replicates) and a p value <0.05 were set as thresholds to identify genes that were differentially expressed between cocultures and monocultures. Upregulation (a positive expression ratio) refers to a higher relative molar concentration of the transcripts of a particular gene of *C. cellulovorans*, *M. Barkeri*, or *M. mazei* in cocultures relative to the respective monocultures, and downregulation (a negative expression ratio) refers to a lower relative molar concentration of transcripts in cocultures. The expression ratios of the differentially expressed genes ([fold change] ≥1.2 and p value <0.05) are shown as heat maps.

**Results**

**Methanogenesis and cellulose utilization**

CH₄ and H₂ production by cocultures of *C. cellulovorans–M. Barkeri* and *C. cellulovorans–M. mazei* and
by monocultures of *C. cellulovorans* were examined by amending 3 g/L of cellulose as the sole substrate. Coculturing *C. cellulovorans* with either *M. barkeri* or *M. mazei* both led to methanogenesis during the cultivation period, and linear CH$_4$ production was observed from day 2 onwards (Fig. 1a). The total duration of CH$_4$ production for *C. cellulovorans*–*M. barkeri* was longer than that for *C. cellulovorans*–*M. mazei* (17 versus 10 days), and the final total amount of CH$_4$ produced in *C. cellulovorans*–*M. barkeri* was substantially higher than that produced in *C. cellulovorans*–*M. mazei* (1.5 ± 0.07 mmol versus 0.7 ± 0.09 mmol). Monocultures of *M. barkeri* and *M. mazei* grown on 50 mM acetate produced 2.8 ± 0.1 mmol and 2.5 ± 0.09 mmol of CH$_4$ respectively (Additional file 1: Figure S1). On the other hand, 0.3 ± 0.01 mmol of CH$_4$ accumulated in monocultures of *M. barkeri* grown with 10 mM formate, 3 mM acetate, and 0.95 mmol H$_2$, whereas 0.2 ± 0.01 mmol of CH$_4$ accumulated in monocultures of *M. barkeri* grown with 3 mM acetate and 0.95 mmol H$_2$. In these monocultures, H$_2$ was depleted gradually during the cultivation period (Additional file 1: Figures S2, S3). No CH$_4$ was observed in the monocultures of *M. barkeri* grown with formate alone. As opposed to CH$_4$ production, 1.5 ± 0.08 mmol of H$_2$ accumulated in monocultures of *C. cellulovorans*, whereas no H$_2$ was detected in the cocultures throughout the cultivation period.

Cellulose degradation was observed in both cocultures and monocultures of *C. cellulovorans*. Coculturing *C. cellulovorans* with either *M. barkeri* (+13.8%) or *M. mazei* (+8.9%) both resulted in enhanced cellulose degradation relative to monocultures of *C. cellulovorans* (Fig. 1b). At the end of the incubation period, a small amount of cellulose remained. This is likely due to the acidic pH, as a result of volatile fatty acids (VFAs) accumulation, inhibiting the metabolic activity of the dark fermenters [35]. No growth or cellulose degradation was observed in the controls of *M. barkeri* and *M. mazei* monocultures grown on cellulose. Corresponding to the higher CH$_4$ production and more complete cellulose degradation, the CH$_4$ yield and production rate of *C. cellulovorans*–*M. barkeri* were nearly two times higher than those of *C. cellulovorans*–*M. mazei* after taking into account the amount of cellulose consumed (Table 1). Together, these results indicate that coculturing *C. cellulovorans* with either *M. barkeri* or *M. mazei* not only enabled CH$_4$ production from cellulose but also enhanced cellulose degradation in comparison to monocultures of *C. cellulovorans*. *C. cellulovorans*–*M. barkeri* exhibited a stronger ability of methanogenesis than *C. cellulovorans*–*M. mazei* in terms of CH$_4$ yield and transformation rate.

**Fermentation products and pH**

Monocultures of *C. cellulovorans* mainly fermented cellulose to H$_2$, CO$_2$, and VFAs, including formate, acetate, butyrate, and lactate (Additional file 1: Table S3). Production of VFAs by *C. cellulovorans* monocultures increased gradually to maximum accumulated concentrations of 9.1 ± 0.4 mM of formate, 3.0 ± 0.2 mM of acetate, 9.0 ± 0.3 mM of butyrate, and 3.6 ± 0.1 mM of lactate (Fig. 2). However, the presence of methanogens in cocultures led to VFAs consumption and induced a change in the fermentation pattern of *C. cellulovorans*, resulting in different VFA concentrations in cocultures relative to

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**Fig. 1** a Comparison of H$_2$ and CH$_4$ production and b cellulose degradation for cocultures of *C. cellulovorans*–*M. barkeri* (CC-MB) and *C. cellulovorans*–*M. mazei* (CC-MM) and monocultures of *C. cellulovorans* (CC). Each data point is an average of biological triplicate and error bars represent one standard deviation.
the *C. cellulovorans* monocultures. For the cocultures of
*C. cellulovorans*–*M. barkeri*, formate and acetate only
transiently accumulated during the cultivation period.
Formate concentration reached its peak of 2.5 ± 0.2 mM
on day 2, then decreased rapidly until depletion on
day 12 (Fig. 2a). Similarly, the acetate concentration
also decreased progressively until near depletion after
reaching its maximum concentration of 1.7 ± 0.1 mM
on day 6 (Fig. 2b). The depletion of formate and acetate
indicates their utilization via methanogenesis by *M.
barkeri*. In contrast, net reduction in the concentrations
of butyrate and lactate concentrations was not observed
during the cultivation period. However, the final con-
centrations of butyrate and lactate were both lower in

**Table 1** Mass and rate of cellulose consumption, and yield and rate of H₂ and CH₄ production

| Cultures           | Cellulose consumed (mg) | H₂ yield (mol H₂/mol glucose equivalent) | CH₄ yield (mol CH₄/mol glucose equivalent) | Cellulose degradation rate (mg/day) | H₂ production rate (mmol/day) | CH₄ production rate (mmol/day) |
|--------------------|-------------------------|------------------------------------------|------------------------------------------|-----------------------------------|-------------------------------|-------------------------------|
| *C. cellulovorans*  | 235 ± 8                 | 1.02 ± 0.03                              | –                                        | 117 ± 4                           | 0.077 ± 0.004                 | –                             |
| *C. cellulovorans*–*M. barkeri* | 267 ± 16             | –                                        | 0.87 ± 0.02                              | 133 ± 8                           | –                             | 0.074 ± 0.003                 |
| *C. cellulovorans*–*M. mazei*   | 256 ± 7                 | –                                        | 0.44 ± 0.04                              | 128 ± 4                           | –                             | 0.036 ± 0.004                 |

Data are the average (± one standard derivation) of biological triplicate.

**Fig. 2** Comparison of VFA concentrations (**a** formate, **b** acetate, **c** butyrate, **d** lactate) for cocultures of *C. cellulovorans*–*M. barkeri* (CC-MB) and *C. cellulovorans*–*M. mazei* (CC-MM) and monocultures of *C. cellulovorans* (CC). Each data point is an average of biological triplicate and error bars represent one standard deviation.
C. cellulovorans–M. barkeri than in the C. cellulovorans monocultures (Fig. 2c, d). On the other hand, only 1.5 ± 0.4 mM of formate and insignificant amount of acetate were consumed in monocultures of M. barkeri grown with 10 mM formate, 3 mM acetate, and 0.95 mmol H2. Similarly, insignificant amount of acetate was consumed in monocultures of M. barkeri grown with 3 mM acetate and 0.95 mmol H2. (Additional file 1: Figures S2, S3). For the cocultures of C. cellulovorans–M. mazei, neither formate nor acetate depletion was observed during the cultivation period. However, whereas the final formate concentration in C. cellulovorans monocultures, the final acetate concentration in C. cellulovorans–M. mazei was lower than in C. cellulovorans monocultures, the final acetate concentration in C. cellulovorans–M. mazei was higher by 18% (Fig. 2a, b), and the final concentrations of butyrate and lactate were also lower in C. cellulovorans–M. mazei (Fig. 2c, d).

The pH of the C. cellulovorans monocultures decreased rapidly from the initial pH of 7.5 ± 0.02 to 6.1 ± 0.05, but the change in the cocultures of C. cellulovorans–M. barkeri and C. cellulovorans–M. mazei was smaller and more gradual (Fig. 3). The more stabilized pH of the cocultures compared to the C. cellulovorans monocultures is likely due to the consumption of VFAs by the methanogens and the change in fermentation pattern of C. cellulovorans in the cocultures. In addition, corresponding to the depletion of formate and acetate and a lower lactate concentration, the final pH of C. cellulovorans–M. barkeri (pH 6.7 ± 0.06) was higher than that of C. cellulovorans–M. mazei (pH 6.4 ± 0.05).

Cell growth and culture morphology

Cell growth of M. barkeri and M. mazei in cocultures was quantified by qPCR (Fig. 4a), and cell morphology was visualized by SEM (Fig. 4b, c). In cocultures, M. barkeri and M. mazei showed steady growth, reaching the stationary phase by day 8 (Fig. 4a). Compared to the monocultures of M. barkeri and M. mazei grown on acetate, M. barkeri and M. mazei in cocultures grew faster with a shorter lag phase. The cell yields of M. barkeri and M. mazei in cocultures were 3.1 × 10^{13} ± 4.5 × 10^{12} cells/mol of CH4 and 4.3 × 10^{13} ± 7.9 × 10^{12} cells/mol of CH4 respectively, whereas M. barkeri and M. mazei in monocultures were 3.5 × 10^{13} ± 3.2 × 10^{12} cells/mol of CH4 and 3.3 × 10^{13} ± 3.3 × 10^{12} cells/mol of CH4 respectively. C. cellulovorans in both monocultures and cocultures grew to a higher final cell concentration compared to the methanogens. However, unlike the different cell growth rates observed for the methanogens between monocultures and cocultures, the presence of methanogens did not result in significant differences in cell growth for C. cellulovorans between monocultures and cocultures (Fig. 4a). The C. cellulovorans growth yield in monocultures was 2.0 × 10^{13} ± 8.0 × 10^{11} cells/g of cellulose, whereas in cocultures of C. cellulovorans–M. barkeri and C. cellulovorans–M. mazei, it was 2.0 × 10^{12} ± 7.2 × 10^{11} cells/g of cellulose and 1.9 × 10^{12} ± 6.4 × 10^{11} cells/g of cellulose respectively. Interestingly, when grown in cocultures, C. cellulovorans and M. barkeri were observed to form aggregates, whereas C. cellulovorans and M. mazei existed as distinct separate cells (Fig. 4b, c).

Expression of genes in pathways

Based on the physiological results of the cocultures, a number of pathways that are important to the regulation and metabolism of the interactions between C. cellulovorans and M. barkeri or C. cellulovorans and M. mazei were investigated in detail (Figs. 5a, 6a). In order to examine how cells regulate these pathways, 17 C. cellulovorans genes, 13 M. barkeri genes, and 7 M. mazei genes encoding key enzymes within the respective pathways were analyzed by RT-qPCR. The fold changes of cocultures relative to their respective monocultures for these 37 selected genes are shown in Additional file 1: Tables S4, S5, S6. Genes with an absolute value of fold change ≥ 1.2 and a p value <0.05 were considered as differentially expressed to infer the regulation of the pathways (Figs. 5b, 6b).

For C. cellulovorans in the C. cellulovorans–M. barkeri cocultures (Fig. 5a, b), two out of three genes encoding cellulase (Clocel 3359 and Clocel 0912), the gene encoding glycoside hydrolase (Clocel 3111) related to cellulose degradation, and the genes encoding hydrogenase (Hyd) (Clocel 4097, Clocel 3813, and Clocel 1155) related to H2 production were all upregulated. The two genes (Clocel 1684 and Clocel 2840) encoding the pyruvate:ferredoxin oxidoreductase (PFOR) within the pathway of pyruvate
to acetyl coenzyme A (acetyl-CoA) were upregulated. For VFA biosynthesis, the gene (Clocel 1892) encoding acetate kinase for catalyzing pyruvate to acetate was upregulated, whereas the gene (Clocel 3674) encoding butyrate kinase within the pathway of pyruvate to butyrate, the gene (Clocel 1811) encoding formate acetyltransferase within the pathway of pyruvate to formate, and the genes (Clocel 2700 and Clocel 1533) encoding l-lactate dehydrogenase were all downregulated.

For *M. barkeri* in the cocultures, the gene (Mbar A1820) encoding acetate kinase within the acetoclastic pathway, the genes (Mbar A1761, Mbar A0795, Mbar A0931, and Mbar A1095) encoding formylmethanofuran dehydrogenase, the gene (Mbar A0833) encoding formylmethanofuran reductase, and the gene (Mbar A0909) encoding formylmethylmethanofuran synthase were all upregulated.

The key pathways of the interactions are highlighted. The final products of the metabolic pathways are also shown in order to describe the carbon and cellular metabolism in the coculture model. Red and blue color lines represent the upregulated and downregulated pathways, respectively. Green color highlights the resulting effects of cocultivation of *CC* with *MB*. The abbreviations are NADH, reduced form of nicotinamide adenine dinucleotide; NAD+, oxidized form of nicotinamide adenine dinucleotide; Hyd, hydrogenase; Fd, reduced form of ferredoxin; Fdox, oxidized form of ferredoxin; PFOR, pyruvate:ferredoxin oxidoreductase; Acetyl-CoA, acetyl coenzyme A; Acetyl-P, acetyl phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; HS-CoA, coenzyme A; FDH, formate dehydrogenase; Formyl-MFR, a formyl-methanofuran reductase; Formyl-H₄MPT, 5-formyl-tetrahydromethanopterin; Methenyl-H₄MPT, 5-methyl-tetrahydromethanopterin; Methylene-H₄MPT, 5,10-methylene-tetrahydromethanopterin; Methenyl-H₄SPT, 5-methyl-tetrahydrodrosarcinapterin; CH₃-Co(III)FeSP, a methylated corrinoid Fe-S protein; MCR, methyl-coenzyme M reductase.
dehydrogenase and methylene-tetrahydromethanopterin dehydrogenase within the CO₂ reduction pathway, the genes (Mbar A0893–0897) encoding the methyl-coenzyme M reductase (MCR)—which catalyzes the final step in the formation of CH₄—and the genes (Mbar A1561–A1562) encoding formate dehydrogenase (FDH) were all upregulated.

For C. cellulovorans in the C. cellulovorans–M. mazei cocultures (Fig. 6a), the genes encoding cellulase (Clocel 3359, Clocel 0905, and Clocel 0912), glycoside hydrolase (Clocel 3111), and Hyd (Clocel 1158) were all upregulated. The two genes (Clocel 1684 and Clocel 2840) encoding the PFOR were also upregulated. For VFA biosynthesis, the gene (Clocel 1892) encoding acetate kinase and the gene (Clocel 1176) encoding formate acetyltransferase were upregulated, whereas the gene (Clocel 3674) encoding butyrate kinase and the gene (Clocel 1533) encoding l-lactate dehydrogenase were downregulated in the C. cellulovorans–M. mazei cocultures.

For M. mazei in the cocultures, the genes (MM 0495–0496) encoding acetate kinase and phosphate acetyltransferase within the acetyltransferase pathway were upregulated, whereas the gene (Clocel 3674) encoding butyrate kinase and the gene (Clocel 1533) encoding l-lactate dehydrogenase were downregulated in the C. cellulovorans–M. mazei cocultures.

Discussion

Coculturing C. cellulovorans with M. barkeri or M. mazei enabled direct conversion of cellulose to CH₄

Previous studies [25, 36] have shown that the cellulolytic bacterium C. cellulovorans is capable of fermenting cellulose to H₂, CO₂, and VFAs—including formate, acetate, butyrate, and lactate as the major metabolites—whereas genomic and physiological studies [21, 37–39] have demonstrated that M. barkeri and M. mazei possess the ability to couple growth and CH₄ generation by the acetoclastic and the CO₂ reduction pathways. Although M. barkeri has never been reported to utilize formate for growth in pure cultures, the genes (Mbar A1561–A1562) encoding the FDH were all upregulated.

Coculturing C. cellulovorans with M. barkeri or M. mazei enabled direct conversion of cellulose to CH₄

In this study, coculturing C. cellulovorans with either M. barkeri or M. mazei both enabled CH₄ production from cellulose and enhanced cellulose degradation compared to monocultures of C. cellulovorans. Surprisingly, in addition to H₂ and acetate consumption, formate was also consumed in the C. cellulovorans–M. barkeri cocultures.
This demonstrates that M. barkeri is able to simultaneously utilize the three precursors (H2, formate, and acetate) for methanogenesis. Consistent with previous results [21], we observed no growth of M. barkeri in monocultures when grown with 50 mM of formate alone after 9 months. Moreover, we mimicked the conditions of the C. cellulosovorans–M. barkeri cocultures by artificially providing mononucleotides of M. barkeri with all three methanogenesis precursors (10 mM of formate, 3 mM of acetate, and 0.95 mmol of H2). However, without the presence of C. cellulosovorans, neither formate nor acetate consumption was significant (only 1.5 ± 0.4 mM of formate, which is less than 15% of the total amount provided, and insignificant amount of acetate were consumed), though 0.95 mmol of H2 was depleted gradually during the cultivation period (no H2 accumulation was measured in the cocultures) (Additional file 1: Figure S2). The insignificant consumption of acetate might be due to the presence of the added H2 since the 0.95 mmol of added H2 was not exhausted immediately. An inhibitory effect of H2 on the acetoclastic methanogenesis in Methanosarcina spp. has previously been reported [40].

Although the cumulative CH4 production of M. barkeri monocultures grown with the three methanogenesis precursors was higher than M. barkeri monocultures grown with acetate and H2 (0.3 ± 0.01 mmol versus 0.2 ± 0.01 mmol) (Additional file 1: Figures S2, S3), it is significantly less than the C. cellulosovorans–M. barkeri cocultures. The increase in cell density of the M. barkeri monocultures grown with the three methanogenesis precursors over the course of the experiment is also significantly lower than the M. barkeri in the C. cellulosovorans–M. barkeri cocultures (7.5 × 107 ± 2.9 × 106 cells/mL versus 4.3 × 108 ± 6.3 × 107 cells/mL) (Additional file 1: Figure S4). These results indicate that monocultures of M. barkeri did not grow as robustly as the M. barkeri in the cocultures even though similar methanogenesis precursors were provided, suggesting the interactions between C. cellulosovorans and M. barkeri might have provided additional metabolic benefits to M. barkeri besides supplying the three methanogenesis precursors.

The observed physiology of M. barkeri in cocultures is reproducible, but whether or not a specific cellulytic bacterium has to be the coculture partner remains to be determined.

According to the physiological characteristics of the cocultures, a conceptual model that describes the interactions between C. cellulosovorans and M. barkeri or M. mazei has been developed (Figs. 5a, 6a). In the C. cellulosovorans–M. barkeri cocultures, cellulose is first fermented to H2, formate, acetate, butyrate, and lactate by C. cellulosovorans (Fig. 5a). M. barkeri then consumes the acetate, H2, and formate for cell growth and methanogenesis through both the acetoclastic (Eq. 1) and the CO2 reduction pathways with electrons derived from H2 and formate (Eqs. 2 and 3). The dependence of M. barkeri on the metabolites of C. cellulosovorans is supported by the H2, VFAs, and pH data, which showed no H2 accumulation, transient accumulation of formate and acetate, and a more stabilized pH in the cocultures compared to monocultures of C. cellulosovorans. Consistent with these physiological observations, the CO2 reduction pathway of M. barkeri was upregulated (Fig. 5b), supporting that H2 and CO2 were utilized for CH4 production in the C. cellulosovorans–M. barkeri cocultures. Upregulation of the genes encoding the FDH further confirmed that formate was also used as an electron donor for methanogenesis through the CO2 reduction pathway. Unexpectedly, the acetoclastic pathway of M. barkeri was upregulated, suggesting that the CH4 production from acetate in the cocultures was more active than in the M. barkeri monocultures grown on acetate. Furthermore, upregulation of the genes encoding the MCR suggests that overall CH4 formation was enhanced in the C. cellulosovorans–M. barkeri cocultures relative to the M. barkeri monocultures.

\[ \text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2 \]  

(1)

\[ 4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \]  

(2)

\[ 4\text{HCOOH} \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} + 3\text{CO}_2 \]  

(3)
CH₄ formation is elevated in the *C. cellulovorans–M. mazei* cocultures.

**Stabilization of pH by methanogens enhanced cellulose degradation**

Due to the consumption of formate and acetate by *M. barkeri* and the reduction in lactate and butyrate production by *C. cellulovorans*, a more stabilized pH was observed in the *C. cellulovorans–M. barkeri* cocultures than in the *C. cellulovorans* monocultures. Studies [35] have shown that acidic pH can inhibit both the H₂ production and the metabolic activity of dark fermenters. Therefore, the more stabilized pH could potentially improve the cellular metabolism of *C. cellulovorans* in the cocultures. Accordingly, our results showed that both the rate and extent of cellulose degradation were enhanced (Table 1), and the cellulose degradation pathway of *C. cellulovorans* was upregulated in the *C. cellulovorans–M. barkeri* cocultures (Fig. 5b). As a result of a lower accumulated concentration of VFAs, the pH of the *C. cellulovorans–M. mazei* cocultures was also more stabilized than that of the *C. cellulovorans* monocultures. However, the pH of the *C. cellulovorans–M. mazei* cocultures was lower than that of the *C. cellulovorans–M. barkeri* cocultures as formate and acetate both accumulated in the *C. cellulovorans–M. mazei* cocultures. Cellulose degradation was also enhanced in the *C. cellulovorans–M. mazei* cocultures compared to the *C. cellulovorans* monocultures, but to a smaller extent compared to the *C. cellulovorans–M. barkeri* cocultures. Correspondingly, the cellulose degradation pathway of *C. cellulovorans* was also upregulated in the *C. cellulovorans–M. mazei* cocultures (Fig. 6b). However, the additional cellulose degraded, and the more optimal pH did not lead to a significant increase in *C. cellulovorans* cell density in either coculture.

**Simultaneous utilization of three methanogenesis precursors promoted CH₄ yield**

The CH₄ yield of the *C. cellulovorans–M. barkeri* cocultures (0.87 ± 0.02 mol CH₄/mol glucose equivalent) was substantially higher than that of the *C. cellulovorans–M. mazei* cocultures (0.44 ± 0.04 mol CH₄/mol glucose equivalent) and this could be attributed to two causes. First, besides H₂ and acetate, additional electrons can be derived from formate for methanogenesis in the *C. cellulovorans–M. barkeri* cocultures, whereas only H₂ and acetate can be utilized in the *C. cellulovorans–M. mazei* cocultures. Second, compared to the *C. cellulovorans–M. mazei* cocultures, the more stabilized pH of the *C. cellulovorans–M. barkeri* cocultures enabled cellulose degradation to be more complete, which resulted in additional H₂, formate, and acetate being produced and provided additional substrates for CH₄ production.

The simultaneous utilization of H₂, formate, and acetate for methanogenesis in the *C. cellulovorans–M. barkeri* cocultures also has advantages over other reported coculture models, in which only H₂ and formate can be utilized for CH₄ production. For example, Celine et al. [17] incorporated the H₂-consuming methanogens from the human colon with the H₂-producing fibrolytic strains to produce CH₄ from cellulose, obtaining the highest CH₄ yield of 0.33 ± 0.037 mol CH₄/mol glucose equivalent. Bauchop et al. [19] employed the rumen H₂- and formate-utilizing methanogens and the rumen anaerobic fungus for methanogenesis from cellulose, achieving the highest CH₄ yield of 0.59 ± 0.009 mol CH₄/mol glucose equivalent. Both of these CH₄ yields are significantly lower than the yield obtained in the *C. cellulovorans–M. barkeri* cocultures (Table 1).

**Metabolic shifts in *C. cellulovorans* in cocultures**

According to the stoichiometric Eqs. 1 through 3, a theoretical maximum CH₄ production of 0.9 mmol is expected from the acetoclastic and CO₂ reduction pathways based on 0.3 ± 0.02 mmol of acetate, 1.5 ± 0.08 mmol of H₂, and 0.9 ± 0.04 mmol of formate produced in the monocultures of *C. cellulovorans*. However, in the *C. cellulovorans–M. barkeri* cocultures, a final CH₄ amount of 1.5 ± 0.07 mmol was measured, which exceeds the theoretical maximum production by 66% according to the *C. cellulovorans* monocultures. This suggests that the sum of H₂, formate, and acetate produced by *C. cellulovorans* in the *C. cellulovorans–M. barkeri* cocultures exceeds that of the *C. cellulovorans* monocultures.

Because the concentrations of H₂, formate, and acetate measured in the cocultures were determined by both the production rate of *C. cellulovorans* and the consumption rate of *M. barkeri*, it is difficult to accurately calculate the concentration of each metabolite produced. In fact, no H₂ accumulation was measured in the cocultures. Alternatively, the gene expressions of the H₂, formate, and acetate production pathways were examined. Upregulation of the PFOR and Hyd in the cocultures (Fig. 5b) supports that H₂ production of *C. cellulovorans* in the *C. cellulovorans–M. barkeri* cocultures was more active relative to the *C. cellulovorans* monocultures. Concomitant with the enhanced H₂ formation, acetate production and the associated adenosine triphosphate (ATP) synthesis for *C. cellulovorans* might also be elevated as additional acetyl-CoA is generated from the enhanced oxidative decarboxylation of pyruvate. Correspondingly, upregulation of the pyruvate to acetate pathway in the cocultures was observed (Fig. 5b). In contrast, downregulation of the pyruvate to formate pathway suggests that the level of formate production was lower in the cocultures.
On the other hand, the final concentrations of lactate and butyrate in the *C. cellulovorans–M. barkeri* cocultures were lower than in the *C. cellulovorans* monocultures. Correspondingly, the pathways of pyruvate to lactate and butyrate were both downregulated. Based on the gene expression, the increase in H₂ and acetate production together with the decrease in formate, lactate, and butyrate production show that the fermentation pattern of *C. cellulovorans* in the *C. cellulovorans–M. mazei* cocultures shifted relative to the *C. cellulovorans* monocultures. The *C. cellulovorans* cocultures shifted relative to the *C. cellulovorans* monocultures. Correspondingly, the pathways of pyruvate to lactate and butyrate as the obligatory fermentation products from cellulose degradation, *M. barkeri* was able to further utilize H₂, formate, and acetate for methanogenesis by both the CO₂ reduction and acetoclastic pathways. Similar interactions were also observed in the *C. cellulovorans–M. mazei* cocultures, except that formate cannot be utilized by *M. mazei* for CH₄ production. A shift in the fermentation pattern in *C. cellulovorans* was observed in both cocultures and the more stabilized pH promoted cellulose degradation and CH₄ production. This study illustrates that the use of a constructed coculture to convert cellulose biomass to CH₄ is a viable strategy to produce renewable energy, and the interactions between the microbial partners could lead to beneficial outcomes. Understanding the microbial interactions in such an artificial coculture could provide fundamental guidance in engineering synthetic consortia for a more efficient large-scale biomethanation process.

Conclusions

In this study, we report a physiological and molecular investigation of two artificially constructed cocultures utilizing cellulose as the sole carbon substrate. In the cocultures of *C. cellulovorans–M. barkeri*, whereas *C. cellulovorans* produced H₂, formate, acetate, butyrate, and lactate as the obligatory fermentation products from cellulose degradation, *M. barkeri* was able to further utilize H₂, formate, and acetate for methanogenesis by both the CO₂ reduction and acetoclastic pathways. Similar interactions were also observed in the *C. cellulovorans–M. mazei* cocultures, except that formate cannot be utilized by *M. mazei* for CH₄ production. A shift in the fermentation pattern in *C. cellulovorans* was observed in both cocultures and the more stabilized pH promoted cellulose degradation and CH₄ production. This study illustrates that the use of a constructed coculture to convert cellulose biomass to CH₄ is a viable strategy to produce renewable energy, and the interactions between the microbial partners could lead to beneficial outcomes. Understanding the microbial interactions in such an artificial coculture could provide fundamental guidance in engineering synthetic consortia for a more efficient large-scale biomethanation process.

Additional file

**Table S1.** Primer sequences for selected genes in RT-qPCR.

**Table S2.** Primer sequences for internal control genes in RT-qPCR.

**Figure S1.** Acetate concentration and CH₄ production profiles of (a) monocultures of *C. cellulovorans* and (b) monocultures of *M. mazei*.

**Figure S2.** Comparison of the increase in cell density over the course of the experiment. Cell density at time zero was about 3.0 × 10⁷ cells/mL for the experiments.

**Table S5.** Comparison of the increase in cell density over the course of the experiment. Cell density at time zero was about 3.0 × 10⁷ cells/mL for the experiments.

**Table S6.** Gene expression of the selected *C. cellulovorans* (CC) genes in response to cocultivation with *M. barkeri* (MB) at mid-exponential growth phase.

**Table S7.** Gene expression of the selected *M. barkeri* (MB) genes in response to cocultivation with *C. cellulovorans* (CC) at mid-exponential growth phase.

**Figure S4.** Comparison of the increase in cell density over the course of the experiment. Cell density at time zero was about 3.0 × 10⁷ cells/mL for the experiments.

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acetyl coenzyme A; acetyl-P; acetyl phosphate; MCR: methyl-coenzyme M reductase; FDH: formate dehydrogenase; ATP: adenosine triphosphate; ADP: adenosine diphosphate; FADH₂: flavin-adenine dinucleotide; Fd⁺: reduced form of ferredoxin; Fd⁻: oxidized form of ferredoxin; H₅-CoA: coenzyme A; Formyl-MFR: a formylmethanofuran; Formyl-H₄MPT: 5-formyl-tetrahydro-methanopterin; Methenyl-H₄MPT: 5,10-methenyl-tetrahydro-methanopterin; Methylen-H₄MPT: 5,10-methylene-tetrahydro-methanopterin; Methyll-H₄MPT: 5-methyl-tetrahydro-methanopterin; Methenyl-H₄MPT: 5,10-methenyl-tetrahydro-methanopterin; Methyll-H₄MPT: 5-methyl-tetrahydro-methanopterin; CH₃-Co(III)FeSP: a methylated corrinoid Fe-S protein.

**Authors' contributions**

HYL designed the study, performed the experiments, analyzed the data, and wrote the manuscript. SKN carried out some of the data analyses. YJY and MWC performed some of the experiments. PKHL conceived of, designed and supervised, the study, and revised the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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