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Evidence of syntrophic acetate oxidation by *Spirochaetes* during anaerobic methane production

Sang-Hoon Lee [a,1], Jeong-Hoon Park [a,b,1], Sang-Hyoun Kim [c], Byung Jo Yu [b], Jeong-Jun Yoon [b], Hee-Deung Park [a,*]

[a] School of Civil, Environmental and Architectural Engineering, Korea University, Seoul 136-714, South Korea
[b] IT Convergence Materials R&D Group, Chungcheong Regional Division, Korea Institute of Industrial Technology, Chungnam 330-825, South Korea
[c] Department of Environmental Engineering, Daegu University, Gyeongbuk 712-714, South Korea

HIGHLIGHTS

- Anaerobic batch reactors were operated to characterize cluster II *Spirochaetes*.
- Cluster II *Spirochaetes* were positively correlated with hydrogenotrophic methanogens.
- Activity of cluster II *Spirochaetes* was inhibited by high hydrogen partial pressure.
- Cluster II *Spirochaetes* are frequently observed syntrophic acetate oxidizers.

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ABSTRACT

To search for evidence of syntrophic acetate oxidation by cluster II *Spirochaetes* with hydrogenotrophic methanogens, batch reactors seeded with five different anaerobic sludge samples supplemented with acetate as the sole carbon source were operated anaerobically. The changes in abundance of the cluster II *Spirochaetes*, two groups of acetoclastic methanogens (*Methanosaetaceae* and *Methanosarcinaceae*), and two groups of hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*) in the reactors were assessed using qPCR targeting the 16S rRNA genes of each group. Increase in the cluster II *Spirochaetes* (9.0 ± 0.4-fold) was positively correlated with increase in hydrogenotrophic methanogens, especially *Methanomicrobiales* (5.6 ± 1.0-fold), but not with acetoclastic methanogens. In addition, the activity of the cluster II *Spirochaetes* decreased (4.6 ± 0.1-fold) in response to high hydrogen partial pressure, but their activity was restored after consumption of hydrogen by the hydrogenotrophic methanogens. These results strongly suggest that the cluster II *Spirochaetes* are involved in syntrophic acetate oxidation in anaerobic digesters.

1. Introduction

Anaerobic digestion is an important process for treating organic wastes such as municipal sludge, livestock wastewater, and high-strength wastewater under anaerobic conditions (Metcalf and Eddy, 2003; Rittmann and McCarty, 2001). This method utilizes a microbial consortium within the domains of bacteria and archaea. Through the activities of the two domains of microorganisms, the organic wastes are ultimately transformed into methane gas. Briefly, bacteria mainly produce acetate and hydrogen via metabolic activities such as disintegration, hydrolysis, acidogenesis, and acetogenesis, performed in series. In turn, the archaea transform acetate and hydrogen into methane through the metabolic activities of acetoclastic and hydrogenotrophic methanogenesis, respectively.

Acetate produced by the bacterial degradation of organic matter can also be metabolized into hydrogen. Although this reaction is thermodynamically unfavorable under standard conditions ($CH_3COO^- + 4H_2O \rightarrow 4H_2 + 2HCO_3^- + H^+$; $\Delta G^{o\prime} = +105$ kJ), the reaction can occur via syntrophic interaction between acetate-oxidizing bacteria and methane producing hydrogenotrophic archaea (Hattori, 2008; Karakashev et al., 2006). Syntrophic acetate oxidation has been investigated in various anaerobic digesters (Cord-Ruwisch et al., 1998; Schnurer et al., 1996, 1999; Zinder, 1994;

* Corresponding author at: School of Civil, Environmental and Architectural Engineering, Korea University, Anam-Dong, Seongbuk-Gu, Seoul 136-713, South Korea. Tel.: +82 2 3290 4861; fax: +82 2 928 7656.
E-mail address: heedeung@korea.ac.kr (H.-D. Park).
[1] These authors contributed equally to this study.

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Zinder and Koch, 1984), and was reported to be facilitated by the presence of high temperature, salts, volatile fatty acids, and ammonia (Schnurer et al., 1999). Several syntrophic acetate-oxidizing bacteria have been reported, including acetate-oxidizing rod-shaped bacterium (AOR), *Clostridium ultunense*, *Themacetogenium phaeum*, and *Thermotoga lettingae* (Hattori, 2008). Among them, strain AOR, *C. ultunense*, and *T. phaeum* were originally isolated from methanogenic anaerobic digesters, while *T. lettingae* was recovered from a sulfate reducing bioreactor (Hattori, 2008). Observation and isolation of syntrophic acetate-oxidizing bacteria from anaerobic digesters suggests that methane formation via syntrophic acetate oxidation appears to be common in anaerobic digesters.

Spirochaetes are frequently found in anaerobic digesters, as well as in natural and engineered anaerobic environments. Typically, they have a helical coiled morphology and grow chemoheterotrophically. Nevertheless, their ecophysiological functions in anaerobic digesters have remained unclear. They have been reported to have metabolic activities including acetate, ethanol, and lactate fermentations from glucose (Godon et al., 1997), as well as acetate oxidation (Lee et al., 2013). Lee et al. (2013) reported that *Spirochaetes* constituted 1.3–30% of the total bacteria in anaerobic digesters used for the treatment of municipal sludge, and proposed that cluster II *Spirochaetes* might carry out acetate oxidation syntrophically with hydrogenotrophic methanogenic archaea. They observed increased activity of the cluster II *Spirochaetes* in response to the addition of acetate into an anaerobic batch reactor seeded with anaerobic digester sludge, which provided the basis for the speculation about syntrophic acetate oxidation. However, the claim was not supported by the enhanced activity of hydrogenotrophic methanogenic archaea during acetate degradation by the cluster II *Spirochaetes*.

The primary objective of the present study was to explore the evidence of acetate oxidation via syntrophic interaction between the cluster II *Spirochaetes* and hydrogenotrophic methanogens by investigating the correlations between the two groups of microorganisms. To this end, the abundances of *Spirochaetes* and four groups of methanogenic archaea were quantified using qPCR analysis based on the 16S rRNA genes from batch reactors supplemented with acetate and the relationships between the cluster II *Spirochaetes* and the acetoclastic and hydrogenotrophic methanogenic archaea groups were analyzed. Moreover, to verify the relationship between the cluster II *Spirochaetes* and hydrogenotrophic methanogens, the activity of each group in response to high hydrogen partial pressure was investigated via reverse transcription qPCR (RT-qPCR) analyses.

2. Methods

2.1. Preparation of inocula for batch reactors

Five anaerobic batch reactors were set up to investigate the correlations between *Spirochaetes* and hydrogenotrophic methanogens. Anaerobic sludge was collected from the five full-scale anaerobic digesters (S1, J2, N1, N2, and T1 digesters), located in Seoul, South Korea. The sludge samples were used as the inocula for the batch test examining methane production. Among them, the S1 and J2 digesters were operated under thermophilic conditions (48–51 °C), while the N1, N2, and T1 digesters were operated under mesophilic conditions (36–40 °C). Detailed information about the digesters and sludge, such as digester configuration, characteristics of feed waste, and operating conditions, were described in a previous study (Lee et al., 2012). Before seeding the inocula into the batch reactors, all sludge samples were starved under anaerobic conditions for four days without the addition of feed at the same temperature conditions as the digesters from which the sludge samples were taken.

2.2. Operation of batch reactors

The reaction mixture (300 ml) for a batch reactor consisted of 30 ml of a starved anaerobic seed sludge, 1,000 mg/l of acetic acid as COD, and 120 ml of 0.1 M carbonate buffer (pH = 7.5). After filling each 500 ml-volume serum bottle with the 300 ml reaction mixture, the bottles were sealed using a rubber stopper, and the head space was purged with pure nitrogen gas (1 L/min of flow rate for 3 min). The sealed serum bottles were then incubated in a shaking incubator (150 rpm) at 35 °C (N1, N2, and T1 inocula) or 55 °C (S1 and J2 inocula) to closely simulate the original operational conditions.

Among the five sludge samples, the T1 sludge was used to investigate the effects of high hydrogen pressure on the activity of cluster II *Spirochaetes*. Two anaerobic batch reactors were prepared using the same reaction mixture conditions described above. The head space of each reactor was purged with pure nitrogen or pure hydrogen gas (1 L/min of flow rate for 3 min), and then sealed using a rubber stopper. Both reactors were operated at 35 °C in a shaking incubator (150 rpm).

2.3. Sampling and analytical methods

Gas samples were periodically collected using a glass syringe for measuring the volume of the methane gas produced. The methane gas contents were measured via gas chromatography (Gow Mac series 580, Bound Brook, NJ, USA) using a thermal conductivity detector (TCD) and a 1.8 m × 3.2 mm stainless-steel column packed with porapak Q (80/100 mesh), employing high purity helium as a carrier gas. The operation temperatures of the injector, detector, and column were maintained at room temperature, 90 and 50 °C, respectively (Park et al., 2011).

To trace the acetic acid concentration during the operation period, 10 ml sludge samples were collected using a disposal syringe after 1, 2, and 6 days of operation. For the measurement of acetic acid, the samples were initially filtrated using 0.45-μm pore-size PVDF membrane filters (Advantec, Tokyo, Japan), after which the filtrate was analyzed via high performance liquid chromatography (HPLC, YL9100 series, Young Lin, Anyang, South Korea) using a refractive index detector (RID), an ultraviolet detector (210 nm), and a 300 × 7.8 mm of Aminex HPX-87H ion exclusion column with 5 mM H_2SO_4 as the mobile phase (Park et al., 2014). The operation temperature of the detector and the column were kept at 35 and 60 °C, respectively.

2.4. DNA/RNA extraction, qPCR, and RT-qPCR

For extraction of DNA and RNA from the batch reactors, the MoBio PowerSoil DNA Isolation kit and MoBio PowerSoil total RNA Isolation kit (Solana Beach, CA, USA) were employed, respectively, following the manufacturer's protocols. Concentrations of the total DNA and RNA extracted were determined spectrophotometrically using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). For both qPCR and RT-qPCR analysis, six primer sets specific for the 16S rRNA gene belonging to the domain *Bacteria*, cluster II *Spirochaetes*, and four groups of methanogenic archaea were used (Table 1). The reactions were conducted using the Bio-Rad CFX-96 real time system (Bio-Rad, Hercules, CA, USA). For qPCR, the reaction mixtures consisted of 10 μl of SYBR Premix Ex Taq™ (Takara, Shiga, Japan), 0.4 μl each of the forward and the reverse primers (10 μM), 0.4 μl of 50 × ROX™ Reference Dye, 2 μl of template DNA, and sterilized deionized water to adjust the final volume of 20 μl. The thermal profile of qPCR for the