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Conductive iron oxides accelerate thermophilic methanogenesis from acetate and propionate

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Anaerobic digester is one of the attractive technologies for treatment of organic wastes and wastewater, while continuous development and improvements on their stable operation with efficient organic removal are required. Particles of conductive iron oxides (e.g., magnetite) are known to facilitate microbial interspecies electron transfer (termed as electric syntrophy). Electric syntrophy has been reported to enhance methanogenic degradation of organic acids by mesophilic communities in soil and anaerobic digester. Here we investigated the effects of supplementation of conductive iron oxides (magnetite) on thermophilic methanogenic microbial communities derived from a thermophilic anaerobic digester. Supplementation of magnetite accelerated methanogenesis from acetate and propionate under thermophilic conditions, while supplementation of ferrihydrite also accelerated methanogenesis from propionate. Microbial community analysis revealed that supplementation of magnetite drastically changed bacterial populations in the methanogenic acetate-degrading cultures, in which *Tepidoanaerobacter* sp. and *Coprothermobacter* sp. dominated. These results suggest that supplementation of magnetite induce electric syntrophy between organic acid-oxidizing bacteria and methanogenic archaea and accelerate methanogenesis even under thermophilic conditions. Findings from this study would provide a possibility for the achievement of stably operating thermophilic anaerobic digestion systems with high efficiency for removal of organics and generation of CH_4.

[**Key words:** Thermophilic methane fermentation; Thermophilic anaerobic digestion; Methanogenic degradation; Methane production; Degradation of organic acid; Magnetite; Conductive iron oxides; Syntrophic relationship; CH_4]

Anaerobic digestion is one of the attractive technologies for the treatment of diverse organic wastes and wastewaters (1). Anaerobic digestion requires less energy investment and generates less excess sludge than the other waste and wastewater treatment approaches such as activated sludge processes (2). Anaerobic digesters operated under thermophilic conditions (typically around 55°C) have numerous advantages over the mesophilic process, including higher digestion efficiency, higher methanogenic rate, and disinfection of pathogenic organisms (3–5). Despite these benefits, thermophilic methanogenic digestion is generally sensitive to environmental fluctuations, including increase in the organic loading rate, accumulation of inhibitory compounds (e.g., ammonia), and decrease in pH (6–9). Relatively lower microbial diversity and higher organic decomposition rate in thermophilic anaerobic digesters are considered to be the major cause of this instability (10,11).

Deterioration of methanogenic digestion process typically results in excessive accumulation of short chain fatty acids, particularly acetate and propionate, indicating that the decomposition of short chain fatty acids is the rate-limiting step of methanogenic digestion (9,12–14). Propionate and acetate are the major intermediates in methanogenic degradation processes produced by hydrolysis and fermentation of complex organic matters. Methanogenic degradation of propionate requires syntrophic interaction of propionate-oxidizing bacteria and hydrogenotrophic methanogens, which is specifically referred to as interspecies electron transfer (IET) (14–16). Propionate-oxidizing bacteria oxidize propionate into acetate and the reducing equivalents are generally consumed by H_2 (and/or formate) production. The produced H_2 is consumed by hydrogenotrophic methanogens as their energy source. Since the anaerobic propionate oxidation reaction performed by propionate-oxidizing bacteria is highly endergonic under the standard conditions and is feasible only under conditions with low H_2 concentrations, H_2-scavenging reaction by hydrogenotrophic methanogens is essential to make the propionate oxidation reaction energetically favorable (14,17,18). Methanogenic acetate degradation potentially proceeds via either direct or syntrophic pathways. In the direct pathway, aceticlastic methanogens convert acetate into CH_4 and CO_2 (19,20). By contrast, in the syntrophic pathway, acetate is degraded via syntrophic interaction between acetate-oxidizing bacteria and hydrogenotrophic bacteria as observed in methanogenic propionate degradation (21,22). While aceticlastic methanogenesis is the primary acetate degradation pathway in mesophilic anaerobic digesters, the syntrophic

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pathway often dominates over the direct pathway in thermophilic anaerobic digesters, especially under stress-inducing conditions (4,22–26). Taken together, the enhancement of IET reaction is crucial for efficient methanogenic degradation of propionate and acetate, necessary for the establishment of stable anaerobic digesters.

Although diffusion of small molecules, including H_2, formate, and quinone compounds, had been considered as the sole mechanism of IET (16,27,28), electric currents flowing through conductive mineral nanoparticles and microbial appendages have recently been proven to mediate IET (29,30). The electric current-mediated IET, termed electric syntrophy, have been reported to mediate IET between fatty acid-oxidizing bacteria and methanogens, by which higher methanogenic rates can be achieved compared to diffusion-dependent IET processes (31–35). Methanogenesis based on electric syntrophy has been confirmed for various mesophilic microbial communities, in which diverse conductive materials, including iron oxides (e.g., magnetite), graphite, and microbial conductive pili, have been reported to work as the electron conduits (31,33,35,36). However, electric syntrophy in thermophilic environments has not yet been observed.

Herein, we investigated whether methanogenic degradation of acetate and propionate by microbial communities derived from a thermophilic anaerobic digester is enhanced by supplementation of conductive iron oxides (magnetite). Microbial community analysis was performed on acetate-degrading microbial communities to assess the effects of conductive iron oxides on the bacterial and archaeal populations.

MATERIALS AND METHODS

Culture and medium Seed sludge was collected from a thermophilic anaerobic digester (4) derived from a methanogenic packed bed reactor at Kajima Technical Research Institute. Seed sludge (1% [v/v] for the acetate culture, 10% [v/v] for the propionate culture) were inoculated into 10 mL of inorganic medium (pH 7.2), containing (L^{-1}) 0.91 g of KH_2PO_4, 2.39 g of $Na_2HPO_4 \cdot 12H_2O$, 0.5 g of NH_4Cl, 0.18 g of $MgCl_2 \cdot 6H_2O$, 5 mL of mineral solution (37), 1 mL of vitamin solution (37), 0.3 g of $Na_2S \cdot 9H_2O$, 0.3 g of cysteine-$HCl \cdot H_2O$, and 1 mg of resazurin sodium salt. Methanogenic substrates (either 20 mM acetate or 25 mM propionate) were supplemented from anaerobic stock solutions. Ferrihydrite, magnetite, and Fe(III)-NTA were synthesized as described elsewhere (38–40) and were supplemented from anaerobic stock solutions. The methanogenic microbial communities were anaerobically cultivated under the gas phase of N_2/CO_2 (80:20 [v/v]) at 55°C under static conditions. All cultivation experiments were conducted in triplicates.

Microbial community analyses Genomic DNA was extracted from culture samples using a direct lysis protocol involving bead beating (41). The clone library analysis and the terminal restriction fragment-length polymorphism (T-RFLP) analysis targeting archaeal and bacterial 16S rRNA genes were performed as described previously (4,42). The nucleotide sequence data obtained in this study have been submitted to GenBank under Accession numbers AB922631-AB922638.

Analytical methods The concentrations of organic acids were measured using a high-pressure liquid chromatograph (Alliance 2695; Waters) equipped with an organic acid analysis system (TSK-GEL OApak-A, P; Tosoh), an UV detector (2996; Waters), and a refractive index detector (2414; Waters). The amount of CH_4 in the headspace was analyzed with a gas chromatograph-mass spectrometer (GC17A-QP5050, Shimadzu) equipped with a CP-PoraPLOT Q (L: 25 m, ID: 0.32 mm, df: 10 μm; GL Science Inc.).

RESULTS AND DISCUSSION

Effect of iron oxides on methanogenic degradation of acetate and propionate Thermophilic methanogenic microbial communities were cultivated on either acetate or propionate as the methanogenic substrates in the presence or absence of either conductive (magnetite) or insulative (ferrihydrite) iron oxides. The concentrations of acetate and propionate were monitored throughout the cultivation (Fig. 1). In the acetate-supplemented Non-Fe control cultures, 20 mM acetate was completely decomposed within 23 days (Fig. 1A). Supplementation of 5 and 10 mM magnetite significantly enhanced the acetate decomposition rate and 20 mM acetate was completely decomposed within 15 days. On the contrary, methanogenic acetate degradation was significantly suppressed and completely inhibited by supplementing 5 and 10 mM ferrihydrite, respectively. Inhibitory effects of readily reducible iron oxides (e.g., ferrihydrite) have frequently been reported for both mesophilic (31,43–45) and thermophilic methanogenic microbial communities (46). Aside from the cultures supplemented with 10 mM ferrihydrite, in which no acetate degradation occurred, all cultures generated a theoretically feasible amount of CH_4 (approximately 20 mmol/l) from 20 mM acetate (Fig. 2A). These results suggested that the conductive property, but not supplementation of iron as micronutrients and/or diffusible electron carrier, is the causative factor of magnetite supplementation for the acceleration of methanogenic degradation of acetate.

As no propionate degradation and methanogenesis were observed for cultures inoculated with 1% (v/v) sludge for more than 100 days (data not shown), higher amount of sludge (10% [v/v]) was

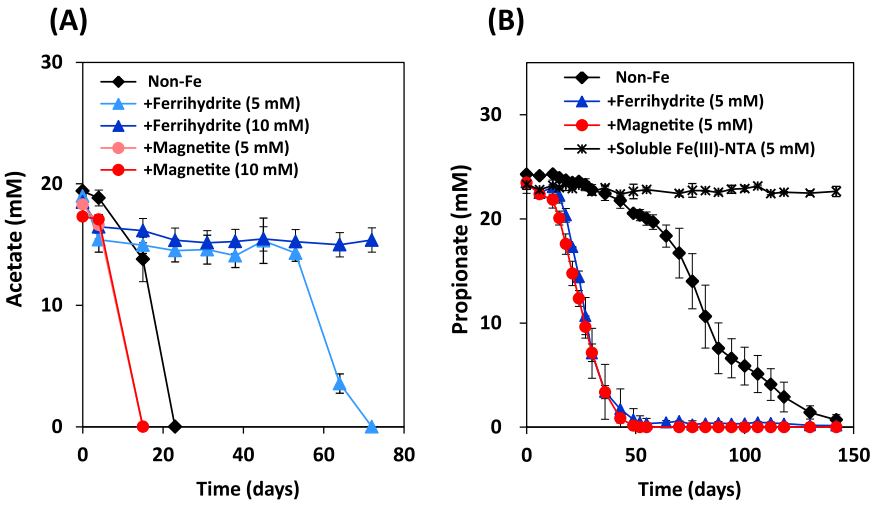

FIG. 1. Effects of magnetite, ferrihydrite and soluble iron, and Fe(III)-NTA on methanogenic degradation of acetate (A) and propionate (B) by thermophilic microbial communities. Data are presented as the means of three independent experiments, and error bars represent standard deviations.