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Conductive properties of methanogenic biofilms

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ABSTRACT

Extracellular electron transfer between syntrophic partners needs to be efficiently maintained in methanogenic environments. Direct extracellular electron transfer *via* electrical current is an alternative to indirect hydrogen transfer but requires construction of conductive extracellular structures. Conductive mechanisms and relationship between conductivity and the community composition in mixed-species methanogenic biofilms are not well understood. The present study investigated conductive behaviors of methanogenic biofilms and examined the correlation between biofilm conductivity and community composition between different anaerobic biofilms enriched from the same inoculum. Highest conductivity observed in methanogenic biofilms was 71.8 ± 4.0 μS/cm. Peak-manner response of conductivity upon changes over a range of electrochemical potentials suggests that electron transfer in methanogenic biofilms occurs through redox driven super-exchange. The strong correlation observed between biofilm conductivity and *Geobacter* spp. in the metabolically diverse anaerobic communities suggests that the efficiency of DEET may provide pressure for microbial communities to select for species that can produce electrical conduits.

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1. Introduction

Methanogenesis is a vital process in regard to biofuel production and the global carbon cycle. Extracellular electron transfer (EET) regulates much of methanogenesis and is of energetic importance in many microbial communities [1,2]. During anaerobic degradation, complex organic matters such as polysaccharides, proteins, and lipids are first oxidized to intracellular intermediates and CO_2 with generation of electrons in the form of reducing equivalents [3,4]. To maintain redox balance, the reducing equivalents have to be utilized by producing fermentation products, which include hydrogen, formate, acetate, lactate, ethanol, propionate, butyrate, *etc.* [3,4]. In methanogenic environments, the further breakdown of these products relies on the syntrophic relationship between methanogenic archaea and syntrophic bacteria which often include *Geobacter* spp. [4–6]. The syntrophic metabolism is regulated through the extracellular transfer of electrons between these two groups of microorganisms [3,4]. The most well-established means of transferring reducing equivalents (electrons) in methanogenic communities is through the indirect exchange of hydrogen/formate. In several systems this is believed to be the rate-limiting step for methanogenesis [7,8]. Recently, alternative direct transfer models of the EET process have emerged, and their existence may enable transfer of electrons as electrical current at high efficiency [5,6,9,10].

In order to transfer electrons directly in the form of electrical current, microbes need to establish electrical connections with extracellular electron acceptors. This physical adaption is evident from construction of conductive extracellular structures, as has been observed in the aggregates of two *Geobacter* species [11]. The detailed conduction mechanisms of conductive microbial assemblages are still under investigation and diverged into two distinct theories, the model of redox conductivity [12–14] and the model of metallic-like conductivity [15–18].

Electrical conductivity is a recognized property of many methanogenic granules [19,20]. *Ex situ* investigation of granule conductivity upon changes of temperature suggests the existence of "metallic-like" conductivity in these granules, though dehydration of granules and bias towards measured area might affect the appropriate elucidation of conductive mechanism [12]. Direct *in situ* investigation of conductive mechanism in methanogenic granules and other types of methanogenic assemblages such as biofilm, that might potentially avoid those misinterpretations, has not previously been conducted.

Additionally, the relationship between the community composition and conductivity in anaerobic mixed-species assemblages (biofilms and granules) has not been elucidated. In both methanogenic and exoelectrogenic communities, *Geobacter* spp. are often highly metabolically active, however, microbial assemblages originating from these communities often possess conductivities varying by orders of magnitude [19–22]. For example, conductivity values of 250 and 680 μS/cm have been respectively reported in exoelectrogenic anodic biofilms containing 52% and 16% *Geobacter* spp., while electrical conductivity of methanogenic granules has been observed in the range of 0.8 to 36.7 μS/cm (2–29% *Geobacter* spp. among bacterial population) [19–22]. As the

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future enhancement of methane-related bioenergy strategies such as anaerobic digester and methane microbial electrolysis cells (MECs) could depend on the elucidation and optimization of methanogenesis *via* DEET [5,10,19,23], conductive mechanism and relationship between conductivity and community membership in microbial biofilms needs to be further investigated.

In the present study, methanogenic biofilms, another form of methanogenic assemblages that possess similar metabolic activities to granules, were examined in *in situ* conditions using a gold-coated split-anode design adapted from previous studies [18,21]. Exoelectrogenic and fermentative biofilms enriched from the same inoculum were used for comparison. The current producing capability of mature methanogenic biofilms was also examined. All communities were sequenced and analyzed in order to determine the relationship between conductivity and community structure. Results provide evidence for a redox driven conductivity mechanism in methanogenic biofilms and suggest important roles in regards to methanogenic community conductivity for exoelectrogens such as *Geobacter* spp.

2. Materials and methods

2.1. Preparation of split electrodes

A split-electrode design modified from a previous study [18] was used for anaerobic biofilm development and *in situ* measurement of biofilm conductivity (SI Fig. 1). We chose to use this split-electrode design in order to ensure the proper contact between the slow growing methanogenic biofilms and the electrode. A water resistant adhesive (Loctite, Düsseldorf, Germany) was pasted to standard weighing paper (Schleicher & Schuell, Inc., Keene, NH, USA) to provide rigidity. Adhesive laden paper was then cut into circle with area of 7 cm^2, and an electrically conductive gold film (approx. 5 μm) was applied to the adhesive layer by an Cressington 108 Auto sputter coater (Cressington Scientific, Watford, UK). The gold coated surface was then cut down at the center by an ESI 5330 UV Laser machine (Electro Scientific Industries, Inc., Portland, OR, USA) to create a 50 μm non-conductive gap. Resistance measurements confirmed that two pieces of electrodes were electrically well separated.

2.2. Biofilm development

Anaerobic sludge collected from the Corvallis Wastewater Treatment Plant (Corvallis, OR) was filtered to remove the large solid particles and used as inoculum for developing anaerobic biofilms. Methanogenic and fermentative biofilms were developed in continuous-flow reactors (12 ml liquid volume) that did not contain cathodes (SI Fig. 1A). The methanogenic reactors were fed with a modified medium solution amended with 50 mM phosphate buffer [20] with sodium acetate trihydrate (1.8 g/l), sodium propionate (0.72 g/l), and ethanol (1.96 g/l) as the carbon source with pH maintained around 7.0. The fermentative reactors were fed with the same medium containing except with glucose as carbon source (9 g/l) and the pH was maintained around 5 to inhibit methane production. The flow rates for methanogenic and fermentative reactors were 0.1 ml/min and 2.8 ml/min, respectively. The exoelectrognenic biofilms were developed on the anode of single-chamber air-cathode microbial fuel cells (MFC) as reported previously [21,24]. The MFCs were operated in fed-batch mode using the same medium solution for methanogenic reactors with external resistance gradually decreased from 10,000 to 500 Ω in between batches in order to maintain the maximum cell voltages around 0.3 V. When voltages were under 5% of batch maximum, medium was replaced with fresh medium. Abiotic control reactors were operated in the same manner as methanogenic reactors but without inoculation. All experiments were conducted in triplicate at 37.0 °C. Methane production was monitored using a gas chromatography (Agilent, 6890N; J&W Scientific, USA) equipped with a thermal conductivity detector with Argon as the carrier gas.

Maturation of biofilms was estimated following suggestions from previous studies [21,25,26]. Fermentative biofilms were considered to reach their maturity following 17 days of operation [25]. Exoelectrogenic and methanogenic biofilms were considered to reach their maturity at 85 and 122 days, respectively [21,26]. Two-electrode method was used to *in situ* measure the conductivity change during the biofilm development. Conductivity was assumed to remain stable following community maturation. The two-electrode conductivity experiments involving fermentative and exoelectrogenic biofilms were ended after biofilm maturation while conductive mechanisms of methanogenic communities were then analyzed through electrochemical gating analysis (day 122). One of the methanogenic samples and all fermentative and exoelectrogenic samples after conductivity analysis were then subject to microscopic and genetic analysis. The rest of the methanogenic biofilms were placed into single-chamber air-cathode MFCs to serve as anodes to further investigate electron exchange capabilities within the methanogenic biofilms. Converted methanogenic MFCs were fed with sodium acetate trihydrate (1.8 g/l), sodium propionate (0.72 g/l), and ethanol (1.96 g/l) were operated for 40 days under fed-batch mode till no change of conductance can be observed and then subjected to genetic analysis.

2.3. Confocal laser scanning microscopy (CLSM)

Following maturation, reactors were opened and small pieces of biofilm-containing electrodes (approximately 15 to 30% of the total surface area) were carefully cut out for CLSM analysis. The rest of the biofilm samples were used for DNA extraction. For the CLSM analysis, specimens containing biofilms were first stained with LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) following the instructions of manufacturer. The stained biofilms were then examined with the Zeiss LSM 780 NLO confocal microscope with a 10× objective lens (Carl Zeiss AG, Oberkochen, Germany), and a minimum of three fields were imaged. Images were further processed and analyzed by software ImageJ (ver. 1.49d) to estimate the thickness of biofilm. A minimum of ten random CLSM stacks along the y axis were used to determine the average thickness of biofilm. The thickness of fermentative biofilm in the gap area was estimated by using fractional caliper due to the excess thickness of biofilms compared to methanogenic and exoelectrogenic biofilms.

2.4. Conductivity measurement (two-electrode method)

To measure the *in situ* conductivity of biofilms over time, two-electrode method was used as described previously [21]. A voltage ramp (0–0.075 V) that is low enough to avoid the electrolysis of water or a self-heating effect was applied between two sides of a gapped electrode in steps of 0.025 V by using a source meter (Model 2401, Keithley, USA). For each voltage step, transient ionic current related to the pseudo-capacitance charging and macro transport of counterions was allowed to decay until a steady state was reached. Current was then measured every 30 s over a 3-min period. For measuring the conductivity of exoelectrogenic biofilms, MFC anodes were temporarily disconnected from the cathode and allowed open circuit potential (OCP) to be reached. Resistances were calculated by plotting average currents of each step and applied voltages to calculate the slope of current-voltage linear curve [14].

End biofilm conductivity was measured at 17 days for the fermentative community, 122 days for methanogenic, 85 days for exoelectrogenic communities, and 40 days following the switch from methanogenic to exoelectrogenic settings for the converted community (162 days total), and 60 days for the abiotic controls. Biofilm conductivity (σ) was calculated by using conformal mapping as suggested in previous study. R is the measured resistance prior to the CLSM examination, D is diameter of

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