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Operation-driven heterogeneity and overlooked feed-associated populations in global anaerobic digester microbiome

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ABSTRACT

Anaerobic digester (AD) microbiomes harbor complex, interacting microbial populations to achieve biomass reduction and biogas production, however how they are influenced by operating conditions and feed sludge microorganisms remain unclear. These were addressed by analyzing the microbial communities of 90 full-scale digesters at 51 municipal wastewater treatment plants from five countries. Heterogeneity detected in community structures suggested that no single AD microbiome could be defined. Instead, the AD microbiomes were classified into eight clusters driven by operating conditions (*e.g.*, pretreatment, temperature range, and salinity), whereas geographic location of the digesters did not have significant impacts. Comparing digesters populations with those present in the corresponding feed sludge led to the identification of a hitherto overlooked feed-associated microbial group (*i.e.*, the residue populations). They accounted for up to 21.4% of total sequences in ADs operated at low temperature, presumably due to ineffective digestion, and as low as 0.8% in ADs with pretreatment. Within each cluster, a core microbiome was defined, including methanogens, syntrophic metabolizers, fermenters, and the newly described residue populations. Our work provides insights into the key factors shaping full-scale AD microbiomes in a global scale, and draws attentions to the overlooked residue populations.

1. Introduction

Wastewater treatment processes, including primary treatment for solids separation and secondary treatment for carbon and nutrients removal, produce substantial amount of waste sewage sludge. For example, the amount of waste sludge generated in European Union is estimated to exceed 13 million dry solid tons in 2020 (Kelessidis and Stasinakis, 2012). Anaerobic digestion (AD) has been used worldwide to simultaneously degrade waste sludge and produce methane, and is a promising solution to treat the increasing global production of organic solid wastes (Appels et al., 2011). Meanwhile, the microbial community involved in AD is complex (Narihiro et al., 2015) and a better understanding of the AD ecosystem would optimize existing processes and enhance the engineering application (Vanwonterghem et al., 2014).

To identify critical populations responsible for the AD process, multiple researches have tried to define the core AD microbiome. Campanaro et al., (2016). and Treu et al., (2016). analyzed metagenomic sequences of mesophilic and thermophilic lab-scale digesters treating cattle manure, and concluded that 77 out of 265 genome bins could be considered as the core essential microbial groups in biogas production. Our recent study analyzed the microbial communities of three full-scale digesters in the a wastewater treatment plant and observed a core microbiome that

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accounted for 59% of the total 16S rRNA gene sequences (Mei et al., 2016a). Studies investigating multiple full-scale plants reported that core populations constituted 36.4% of the total 16S rRNA gene sequences in seven digesters from Seoul, South Korea (Lee et al., 2012), and 28% of the total 16S rRNA gene sequences in seven digesters from France, Germany, and Chile (Riviere et al., 2009). De Vrieze et al. (De Vrieze et al., 2015) evaluated the microbial communities of 29 AD installations whose locations were not specified, and reported that *Clostridiales* and *Bacteroidales* were part of the core microbiome as they were shared by each sample with >0.1% abundance. So, if a large number of digesters are sampled and multiple operating parameters are considered, such as temperature, ammonia concentration, and system configuration that are known to influence AD community (De Vrieze et al., 2015; Smith et al., 2017), would it be still possible to define a core AD microbiome? Furthermore, geographical differences in microbiomes have been observed for waste-treating ecosystems like activated sludge (Zhang et al., 2012) and solid waste landfill (Stamps et al., 2016). Would a similar difference be observed with the AD microbiome?

A classic categorization of microorganisms in AD consists of fermenting bacteria (fermenters), syntrophic metabolizers (syntrophs), and methanogenic archaea (methanogens) (Schink and Stams, 2006). However, it has been realized that AD microbiome embraces a large proportion of prokaryotes with unrecognized ecophysiology (Narihiro, 2016). For example, our recent study (Mei et al., 2016a) revealed that 25% of the AD populations in one wastewater treatment plant migrated from the upstream activated sludge process and remained as residue populations in AD. The presence of those non-anaerobic residue populations has not been widely examined to test whether it is a common phenomenon in all digesters under different operating conditions from different geographical locations. Furthermore, the microbial populations in activated sludge can vary considerably due to differences in process configuration and geographical locations (Zhang et al., 2012). Thus, it is not clear whether such variations of microbial populations in the feed sludge impact the AD microbiome.

In this study, we used high-throughput sequencing technologies to characterize microbiomes in digesters around the world by sampling 90 full-scale digesters with diverse operating conditions and feed sludge characteristics from 51 municipal wastewater treatment plants. The impacts of operating conditions and geographical locations on AD microbiome were examined. Clustering of samples was performed and cluster-specific core populations were identified. Within the AD microbiome, feed-derived populations were investigated and the distribution in different digesters was characterized.

2. Materials and methods

2.1. Sample collection

In total, 148 digester sludge samples were collected from 90 full-scale ADs in 51 municipal wastewater treatment plants. Feed sludge in 27 plants were collected prior to entering ADs, and feed sludge in the rest of plants were not collected due to sampling difficulties. All operation-related information was provided by the plant operators. Besides the volatile solids reduction (VSR) provided by plant operators, we calculated VSR values using the Van Kleeck equation according to the USEPA regulation (Regulations, 2003), which were further used in the downstream analyses. Most plants were operated with the conventional primary-secondary (activated sludge) treatment scheme, while three plants were only configured with primary treatment before AD (plant CAII, CALG, and USRA). Seven plants (JPHW, JPMU, JPNA, JPST, JPTB, JPYS, and USDV) used a two-stage anaerobic digestion process with similar sludge retention time (the first digester treating sludge from primary/secondary clarifiers and the second digester treating sludge from the first digester). Seven plants (JPHG, JPNA, JPNG, USST, USUR, NEAV, and USCA) introduced external solid wastes into digesters, such as food waste, green waste, and sludge from other sources. Wastewater to two Hong Kong plants (HKST and HKTP) had approximately 1/4 to 1/5 of seawater of high salinity. Due to its high saline nature with high sulfate content, these two AD digesters dosed ferric chloride ($FeCl_3$) to suppress sulfide production, leading to a chloride concentration of 4000 to 6000 mg/L (Koenig and Bari, 2001; Zhang et al., 2012). Wastewater to another Hong Kong plant (HKYL) had effluent from the tannery industry and contained high concentrations of Zn and Cr (Wong et al., 2001). Digester NEAV1 had both high salinity influent (electrical conductivity about 30–35 mS/cm) and external food waste sludge simultaneously. Digesters from Hong Kong and US (except for USWA and USSF) were sampled at multiple time points with at least one-month interval. These multiple time points samples were considered as different samples. Fifty milliliters of sludge were collected from the recirculation lines of digesters, transported to laboratory in UIUC on ice (including international samples), and stored at −80 °C until DNA extraction.

2.2. 16S rRNA gene sequencing

Genomic DNA was extracted from 2 mL of well-mixed sludge using the FastDNA SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA), and quantified using a Nanodrop 2000c spectrophotometer. For PCR amplification, 60 ng of genomic DNA was added into a total reaction volume of 25 μL as template. With a dual-indexing approach (Kozich et al., 2013), a universal primer set 515F (5′-GTGCCAGCMGCCGCGGTAA-3′)/909R(5′-CCCCGYCAATTCMTTTRAGT-3′) targeting the V4-V5 region of both bacterial and archaeal 16S rRNA gene was used for PCR amplification. PCR was performed with the thermal cycling protocol consisting of initial denaturation (94 °C, 3 min), 25 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 45 s) and extension (72 °C, 1 min), and a final extension (72 °C, 10 min) (Mei et al., 2016b). The PCR amplicons were purified using the Wizard SV Gel and PCR Clean-Up system (Promega, Fichburg, WI, USA) and quantified by Qubit 2.0 Fluorometer. Library preparation and sequencing on Illumina Miseq Bulk 2 × 300 nt paired-end system was performed at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign, IL, USA.

2.3. Microbial community analyses

Paired-end raw sequences were assembled, screened, and trimmed using Mothur 1.33.3 (Schloss et al., 2009) with a maximum sequence length of 400 bp and a quality score of 20. The output data were analyzed using QIIME 1.9.1 (Caporaso et al., 2010b) for OTU (operational taxonomic unit, 97% sequence similarity) picking with the *de novo* strategy, which included OTU grouping by UCLUST (Edgar, 2010), alignment by PyNAST (Caporaso et al., 2010a), chimera identification by ChimeraSlayer (Haas et al., 2011), taxonomy assignment by BLAST using reference sequences in the GreenGene 2013 database. After removing singletons (OTUs that only had one sequence in the entire dataset), all samples were rarefied to an even depth of 20,957 sequences (determined by the sample with fewest sequences). Shannon index ($H = -\sum p_i ln p_i$, p_i is the relative abundance of an individual population) calculation, UniFrac distance matrix calculation, Bray-Curtis distance matrix calculation, principal coordinate analysis (PCoA), and unweighted