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Metagenomic and bioanalytical insights into quorum sensing of methanogens in anaerobic digestion systems with or without the addition of conductive filter

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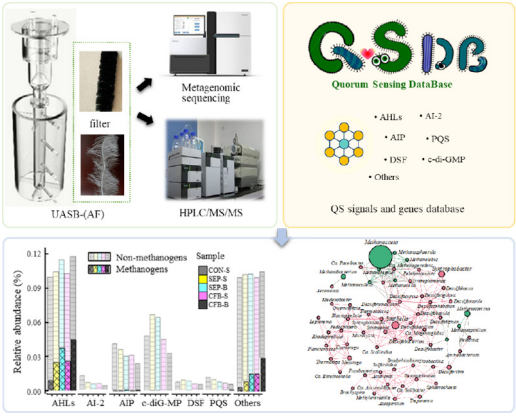
HIGHLIGHTS

- Methane yield was enhanced in the bioreactor with conductive filter.
- A comprehensive and public microbial quorum sensing database was constructed.
- Acyl-homoserine lactone system is the most probable quorum sensing in methanogens.
- The abundant AHLs and AHL-based QS genes coupled with a high methane production
- *Syntrophobacter* and *Smithella* may use RpfB and PDE to communicate with methanogens.

GRAPHICAL ABSTRACT



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ABSTRACT

Understanding microbial interactions in the methanogenesis system through quorum sensing (QS) is very important for system optimization. Known QS genes were collected and classified into seven groups based on the signal molecules, which were used for constructing a hierarchical quorum sensing database (QSDB). QSDB containing 39,981 QS genes of seven QS groups was constructed and QS genes were analyzed with QSDB. Methanogen genomes were aligned with QSDB and acyl-homoserine lactones (AHLs) system was predicted as the most probable QS system. This database was further applied to analyze QS in methanogens from two upflow anaerobic sludge blanket-anaerobic filter hybrid reactors with conductive filter (CFB) and nonconductive filter (SEP), and a control without filter (CON). The maximum COD degradation rates in CFB (722.2 ± 10.1 mg/L·h) was elevated by 42.9% compared to CON (505.4 ± 5.98 mg/L·h). Metagenomic sequencing revealed *Methanosaeta*, *Methanobacterium*, and *Methanosarcina* were dominant, and the abundances was 4.3 times higher in the sludge of CFB compared to CON. The overall abundance of QS genes was CFB > SEP > CON, and AHLs were the most abundant group of QS genes. The filI/filR system, a luxI/luxR homolog, was firstly detected in methanogens, showing a high abundance in the CFB (0.085%) compared to in the CON (0.058%). The concentration of AHL molecules in CFB biofilms (0.04%) was about four times that in the CON (0.01%). *Syntrophobacter* and *Smithella* were the two major syntrophic bacteria of methanogens, and their abundances were positively correlated with methanogens. In addition, *Syntrophobacter* and *Smithella* harbored QS RpfB (component of the diffusible signal factor system) and PDE (component of cyclic di-GMP system). This study provides useful guidance for deeply understanding of QS in anaerobic digestion systems.

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1. Introduction

Anaerobic wastewater treatment is the key strategy for achieving energy recovery from wastewater, with advantage of low sludge production (Lim et al., 2020; Speece, 1983). During anaerobic digestion, complex organic substrates are transformed into simple compounds by orchestrated functions of microbial consortia. Typically, bacteria conduct hydrolysis, acidogenesis, and acetogenesis reactions, and intermediates are converted to methane and carbon dioxide by methanogens. To complete the whole process, cross-species or even cross-kingdom syntrophic cooperation, probably via electron transfer, is required. Therefore, the efficiency of anaerobic digestion is relatively low. Accumulating evidence have demonstrated how methanogens and the syntrophic bacteria cooperated in anaerobic bioreactors (Wang and Yin, 2019; Zhang et al., 2016). Recent studies showed that conductive materials could improve methane production (Yin et al., 2018). However, how methanogens and syntrophic microorganisms cooperate, and how they are affected by the conductive materials still require further clarification.

Quorum sensing (QS) is an information communication mechanism utilized by microbes to control and tune population behavior, during which extracellular chemicals are produced and released as the signal molecules (Tang et al., 2019a, 2019b). Multiple behaviors are regulated by QS, such as the formation of biofilm, the formation of granular sludge, and membrane biofouling (Burton et al., 2005; Hu et al., 2016; Ren et al., 2013). Based on the types of signal molecules, three main categories of QS systems are acyl-homoserine lactones (AHLs) for gram-negative bacteria, autoinducing peptides (AIP) for gram-positive bacteria, and autoinducer-2 (AI-2) for microbial interspecies communication. Other types are quinolone-like 2-heptyl-3-hydroxy-4-quinolone (PQS), inter- and intra-species diffusible signal factor (DSF), second messenger cyclic dimeric (3–5) GMP (*c*-di-GMP) and competence stimulating peptide (CSP) (Harshad et al., 2014; Waters and Bassler, 2005).

Signal molecules can be measured to characterize QS in microbes. Currently, signal molecules are mainly detected through the high performance liquid/gas chromatography and mass spectrometry (Cataldi et al., 2007; Fekete et al., 2007; Lépine et al., 2018; Ortori et al., 2011; Sun et al., 2017). However, these methods cannot pinpoint specific microorganisms involved in the QS process. It is also expensive and time-consuming to identify signal molecules in unknown samples. The bioinformatic analysis offers a unique opportunity to reveal potential QS that may be used in a microbial community. Recently, Tang et al. (2018) found that no common AHL-like genes such as LuxI or LuxM but only HdtS existed in the genome of anaerobic ammonia-oxidizing *Candidatus Jettenia caeni*, indicating that their AHL-based QS was performed through HdtS-type AHLs. This was further verified experimentally (Tang et al., 2019b). Therefore, the genomic analysis could provide guidance in the search of signal molecules, with a great potential to be generalized to other less-studied microorganisms.

To date, the QS of methanogens is rarely studied. Zhang et al. (2012) showed that Archaea *Methanosaeta* used carboxylated AHLs, which were synthesized by the Fill gene and were sensed by the FilR gene, different from the three types of AHL synthases in bacteria: LuxI, LuxM/Ains, and Htds. Furthermore, in anaerobic methanogenesis systems, the syntrophic bacteria might utilize DSF and *c*-di-GMP for cell-cell communication, but the QS system used by methanogens was unrevealed (Yin et al., 2020). The types of genes involved in QS within methanogens and between methanogens and bacteria, need to be sorted and systematically analyzed, especially with the application of bioinformatic techniques.

This study aimed to identify QS in anaerobic methanogenesis systems and investigate the roles of QS in methane generation. To guide the search of potential QS genes and QS signal molecules, QS gene databases were constructed and potential QS genes in the genomes of known methanogens were analyzed. Up-flow anaerobic sludge blanket (UASB) reactors, widely used for anaerobic digestion, were then operated to analyze QS in methanogenesis systems under various conditions. Anaerobic filters (AF) were added to enhance biofilm formation and therefore improve biomass concentrations. Since recent studies have shown that conductive material could improve methane production (Yin et al., 2018), the filters made of conductive and non-conductive materials were also compared.

2. Materials and methods

2.1. Quorum sensing gene collection and database construction

The overall workflow of the QS gene collection and database construction is summarized in Fig. 1A. The QS pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (ko:02024) was used as the basis. Genes directly involved in synthesis or sensing of signal molecules were selected, and all amino acid sequences from the selected KEGG Orthologs (KOs) were retrieved (accessed in August 2019). However, the KO-based collection incorrectly included eukaryote genes. Furthermore, new QS genes identified in recent studies were missing in the KEGG database. Therefore, an exhaustive literature search was carried out to find additional QS genes. Their amino acid sequences were retrieved from the NCBI non-redundant (nr) protein database, and then merged with sequences from KEGG. After that, eukaryote genes and redundant sequences were removed.

The QS gene sequences were organized in a hierarchical structure. The genes were divided into seven sub-databases according to the signal molecules used in the system, namely AHLs, AIP, AI-2, PQS, DSF, *c*-di-GMP and Others. In each sub-database, genes were grouped based on their KOs, representing their different roles in QS. The sequences of the genes were provided in the FASTA format, and the source organisms were recorded in the sequence description line. With this design, it is very convenient to accurately sort the identified QS genes into individual subgroups, and trace the microorganism carrying the specific gene. The detailed information on the seven types of QS pathways and genes are listed in Table S1.

2.2. Mining quorum sensing genes in methanogens

In total, 69 complete genomes from 23 species of methanogens in the Archaea kingdom were available in the NCBI genome database (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/archaea). Genomic sequences in the FASTA format, and gene annotation information in the GenBank format, were downloaded for each genome. Taxonomic lineages of these methanogens were retrieved from the NCBI taxonomy database (ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy/taxdump.tar.gz). Amino acid sequences of the genes in each genome were extracted and aligned to the QS database (QSDB) using BLASTP. A gene was considered as a potential QS gene if it had over 40% identity to at least one gene in QSDB with the BLAST *E*-value below 10^{-4}. The presence of genes in the acetoclastic, hydrogenotrophic and methylotrophic methanogenesis pathways, as annotated in the KEGG database, was summarized to group the methanogens (Table S2).

Since methanogens may interact with other archaea and bacteria in the methanogenesis systems via QS, QS genes in other microbial genomes were analyzed. In total, 11,255 complete bacterial genomes and 303 complete archaea (including methanogens) genomes were obtained from the NCBI genome database (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/) and aligned to QSDB using the same strategy as the methanogens.

2.3. Setup and operation of anaerobic methanogenesis reactors

Two UASB-AF hybrid systems and a UASB reactor were constructed using cylindrical glass with a volume of 6 L and a height of 1.6 m. Carbon fiber brush (conductive material, Haotexin Materials, China, the carbon content of 93%) and solid elastic packing (non-conductive material,

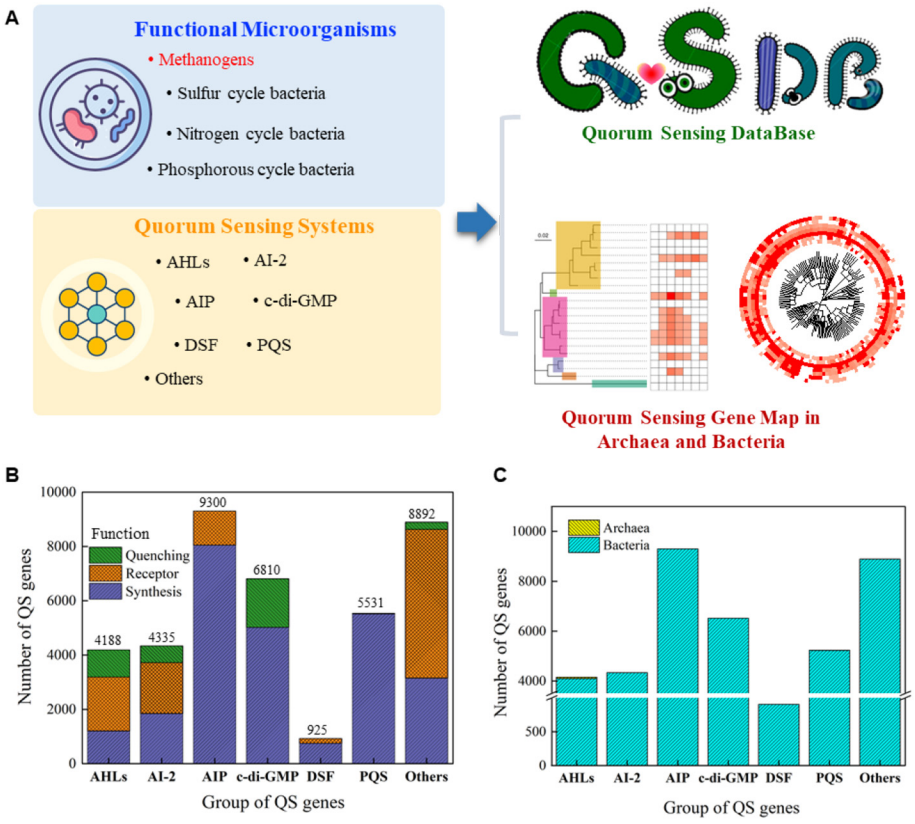

Fig. 1. Construction of the database, and the number of quorum sensing (QS) genes in the database. (A) Schematic diagram of QS gene collection and database construction. QS genes were divided into seven groups based on the signal molecules (AHLs, AI-2, AIP, *c*-di-GMP, DSF, PQS and Others); (B) Number of synthesis, receptor and quenching genes in each QS group; (C) Host (bacteria or archaea) of the QS genes in QSDB.

purchased from Kepu, China and tailored to the same apparent size as the carbon fiber brush) of 70 cm*Ø3.5 cm were placed into two hybrid reactors, referred to as CFB and SEP, respectively. The control reactor was installed without the filter and named as CON. Continuous flow with external circulation was adopted in all three reactors under 35 ± 3 °C. The hydraulic retention time was 48 h with the influent flow rate of 250 mL/h, the recycle flow rate of 1900 mL/h and the upflow velocity was 0.57 m/h. The activated sludge was taken from a municipal wastewater treatment plant in Shenzhen, China for biological nutrient removal and stored under anaerobic conditions for several months before inoculating to the bioreactors. The concentration of the inoculum suspended sludge (SS) was 5.09 ± 0.22 g/L and the volatile suspended sludge (VSS) was 2.11 ± 0.18 g/L.

With the chemical oxygen demand (COD) ratio of 1:1:1, ethanol, acetate, and propionate were contained in the synthetic wastewater. In addition, the COD:N:P ratio was 200:5:1 and trace elements were 1 mL/L. The detailed components of the influent, including nitrogen, phosphorous and vitamins, were the same as described in Yin et al. (2018). Initially, with the influent COD concentration of 4500 mg/L, the COD removal efficiency was quite low, so a stepwise escalating strategy was adopted to acclimate the anaerobic microbial consortium in four phases. In each phase the bioreactors were operated until the effluent COD concentration remained below 300 mg/L for at least three consecutive days. In stage I, the influent COD concentration was 1500 mg/L, which was escalated to 2250 mg/L (stage II), 3000 mg/L (stage III) and finally 4200 mg/L (stage IV). On the last day of the operation, sludge samples from three reactors were collected and named CON-S, SEP-S and CFB-S. Additionally, biofilm samples were scraped from the filters of SEP and CFB using sterile blade, named SEP-B and CFB-B, respectively.

The methanogenic activity in three reactors was evaluated by batch experiments. Under steady state, sludge and biofilm biomass were removed from CON, SEP and CFB with 250-mL aliquots and then placed in 500 mL batch glass bottles. The influent was the same as the one used in phase IV of the long-term operation. Before starting the experiment, the bottles were purged with N_2 for 15 min, and next the rubber stoppers were used to seal them. Following, the bottles were mixed at 35 °C and 160 r/min in an air bath shaker (THZ-300C, Bluepard, China). During batch experiment, liquid samples were collected for parameter analysis.

2.4. Analysis methods

The measurement of COD, SS and VSS were followed the standard methods (APHA, 2005). Methane was measured according to Yin et al. (2017). AHL signal molecules were extracted and measured by using the method described by Sun et al. (2017). The *t*-test was used to compare the concentrations of signal molecules in different bioreactors. The normality of the data was confirmed by the Shapiro-Wilk test, and the homogeneity of variances was confirmed by the Fligner-Killeen test.

DNA was extracted from sludge and biofilm biomass by using PowerSoil DNA Extraction Kit. Metagenomic sequencing libraries were constructed by using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA), and sequenced using the Illumina X-ten sequencer, generating about 6 Gb pair-end reads of length 150 bp for each sample. In total, 30 Gb raw reads were generated in this study.

The quality of the raw sequencing reads was assessed with FastQC, and low-quality (average quality <20) reads were cleaned out by running fastp (version 0.20.1) (Chen et al., 2018). The clean reads in all five samples were pooled together and assembled with MEGAHIT (version 1.0.6) with default parameters (Li et al., 2015a). Open reading frames on the resulting scaftigs of length ≥ 500 bp were predicted using MetaGeneMark (version 3.38) (Zhu et al., 2010). Subsequently,

the predicted genes were clustered by running Linclust. The longest gene in each cluster was selected (named as a unigene), forming the gene catalog of the metagenomes. The clean reads in each sample were then aligned to the gene catalog with bbmap to calculate the relative abundance of the genes in each sample (Yin et al., 2020).

To annotate the unigenes, they were aligned to the NCBI non-redundant protein database using DIAMOND with the *E*-value $<10^{-4}$ (Buchfink et al., 2015). Taxonomic classification was conducted with the aligned results by importing to MEGAN using the Lowest Common Ancestor algorithm. The unigenes were also aligned with the KEGG database for functional annotation. Finally, the clean reads were aligned against QSDB to identify and quantify QS genes in each sample. Statistical tests, such as *t*-test, Wilcoxon signed rank test and Pearson correlation test (where specified), were performed using R (version 3.5.3). Statistically significant was considered with the $P < 0.05$. For the co-occurrence network analysis between syntrophic bacteria and methanogens, the Pearson correlation was calculated using R (version 3.5.3), and the genera pairs with $P < 0.05$ and Pearson's coefficient > 0.8 were selected to construct the network. The network was visualized using Gephi software (version 0.9.2).

3. Results and discussion

3.1. Analysis of potential QS genes in methanogenic archaea genomes

The quorum sensing database, QSDB, is publicly available at https://github.com/qhmu/QSDB. As summarized in Fig. 1B, the current version of QSDB contains 39,981 QS entries, covering seven subgroups of QS systems. The AIP system contained the maximum number of entries (9300), followed by *c*-di-GMP (6810), PQS (5531), AI-2 (4335) and AHLs (4188). The DSF system contained the least number of QS entries (925). The remaining QS systems, such as the competence-stimulating peptide (CSP) used by *Streptococcus pneumoniae*, and PhrC pentapeptide used by *Bacillus subtilis*, were assigned to the 'Others' group, and this group contained 8892 genes. A typical QS pathway contains a synthesis gene which is responsible for synthesizing the signal molecule, and a receptor which receives the molecule. In QSDB, the majority of synthesis genes are from the AIP (8048), *c*-di-GMP (5019) and PQS (5511) groups, while AHLs (1985) and AI-2 (1870) groups contain the most receptor genes. Quorum quenching genes interfere and interrupt the QS process by degrading signal molecules or inhibiting their synthesis. QSDB contains 3674 quorum quenching genes, most of which are from the AHLs and *c*-di-GMP groups.

Over 99% of the QS genes in QSDB were from bacteria (Fig. 1C). In archaea, 54 QS genes in the AHLs group, including filI, filR, and ahlD/aiiA/attM/blcC, and several genes in the PQS groups such as phnA and phnB, were collected. In literature a few studies have shed light on QS in archaea, but most of them are phenomenal descriptions. For example, Paggi et al. (2003) found that the extract from late exponential and stationary growth phases of *Natronococcus occultus*, an haloalkaliphile archaeon, could activate an AHL biosensor, indicating that *N. occultus* might produce AHL for cell-cell communication. Tommonaro et al. (2012) isolated small molecules belonging to diketopiperazines from an extremely halophilic archaeon named *Haloterrigena hispanica*, and found that one of the compounds, cyclo-(L-prolyl–L-valine), could activate AHL bioreporters. Both studies supported the hypothesis that AHL might be used by archaea for QS, but the sequences of the proteins or nucleotides responsible for generating these small molecules were unrevealed. A phosphotriesterase-like lactonase in *Vulcanisaeta moutnovskia*, member of the Crenarchaeota phylum, was able to degrade AHLs, but it has not been demonstrated to participate in QS (Kalia et al., 2011). Shimada et al. (2010) resolved the metallo-β-lactamase superfamily protein structure in *Sulfolobus tokodaii*, which was similar to the QS protein PqsE in *Pseudomonas aeruginosa*, but its function in QS was not validated, either.

Overall, our understanding of archaeal QS is still lacking (Rumbaugh and Sauer, 2020; Zhang et al., 2020). The construction of a publicly available QS gene database provides a reference for the search of signal molecules, especially in less-studied microorganisms, including archaea. To identify potential QS genes in methanogens and their syntrophic species, the complete genomes from NCBI RefSeq database were aligned with QSDB. This analysis generated a whole picture of QS genes in both bacteria and archaea (Fig. S1A–B). On average, each microbial genome carries about four QS genes. In bacteria, the majority of QS genes were in the AHLs, AIP and PQS groups, while in archaea the major group was AHLs. Interestingly, sequences similar to *c*-di-GMP QS genes were also observed in archaeal genomes (Fig. S1B).

Bacterial QS genes have been widely studied (Deng et al., 2011; Galloway et al., 2011; Huang et al., 2020; Ren et al., 2013; Robitaille et al., 2020; Toyofuku et al., 2008; Whiteley and Lee, 2015). We propose that archaeal genes homologous to the bacterial QS genes may also be components of QS systems. In a previous study, amino acid segments similar to bacterial AHL-lactonases and AHL-acylase were found in genomes of *Euryarchaeota* and *Crenarchaeota* (Kallnik et al., 2014). Previous analysis also found a diadenylyl cyclase protein domain, similar to the *c*-di-GMP gene, encoded in some euryarchaeal genomes, leading to the speculation that *c*-di-AMP, another cyclic nucleotide derivate, might be the second messenger in archaea (Romling, 2008). Recent studies demonstrated the presence of *c*-di-AMP in *Haloferax volcanii*, a halophilic mesophile archaeon, and suggested the possible osmotic regulation by *c*-di-AMP (Braun et al., 2019). These evidences suggested that similarity-based in silico analysis could provide insight into the potential QS genes in archaea genomes.

Among these 69 methanogens with available complete genomes, many are common methanogens in wastewater treatment systems and biofilms, and 35 methanogens contain genes to utilize both acetic acid and CO_2 but not methanol or methylamine or dimethylamine or trimethylamine for methanogenesis (Fig. 2, Table S2). This included species from the typical acetoclastic methanogen genus, *Methanosaeta*, which was recently demonstrated to utilize CO_2 for about 1/3 of its methanogenesis (Carr et al., 2018; Rotaru et al., 2014). *Methanosaeta harundinacea* contains all the genes encoding the enzymes in the hydrogenotrphic methanogenesis pathway, including formylmethanofuran dehydrogenase (EC: 1.2.7.1), formylmethanofuran-tetrahydromethanopterin N-formyltransferase (EC: 2.3.1.101), methenyltetrahydromethanopterin cyclohydrolase (EC: 3.5.4.27), methylenetetrahydromethanopterin dehydrogenase (EC: 1.5.98.1) and 5,10-methylenetetrahydromethanopterin reductase (EC: 1.5.98.2) and coenzyme F420 hydrogenase (EC: 1.12.98.1). *Methanobacterium formicicum*, a typical hydrogenotrophic methanogen, contains genes of acetyl-CoA synthetase and acetyl-CoA decarbonylase/synthase (ACDS) (www.kegg.jp/dbget-bin/www_bget?mfc:BRM9_0010+BRM9_0799+BRM9_0797+BRM9_0796), and therefore may also have the potential to perform acetoclastic methanogenesis. The rest 34 methanogens, including those of the *Methanosarcina* genus, contain genes in acetic acid, hydrogenotrophic and also methyl-group oxidation pathways thus have the potential to produce methane using all three metabolic pathways.

The most prevalent QS genes in methanogens belongs to the AHLs group. AHL-related QS genes were observed in 75.2% (52/69) of the methanogen genome (Fig. 2, Table S3). There are two types of potential AHLs QS systems in methanogens, namely filI-filR and ahlD/aiiA/attM/blcC. The filI-filR is a homolog of the bacterial luxI/luxR system, and ahlD/aiiA/attM/blcC is a quorum quenching gene encoding N-acyl homoserine lactone hydrolase (Table S1). Methanogens of the *Methanosaeta* genus tend to use the filI/filR system, while *Methanothermobacter* sp. EMTCatA1, *Methanothermobacter thermautotrophicus* and *Methanothrix thermoacetophila* and others are likely to have both (Table S3). Four species in the *Methanobrevibacter* genus can only use the ahlD/aiiA/attM/blcC system. The three prevalent methanogens in

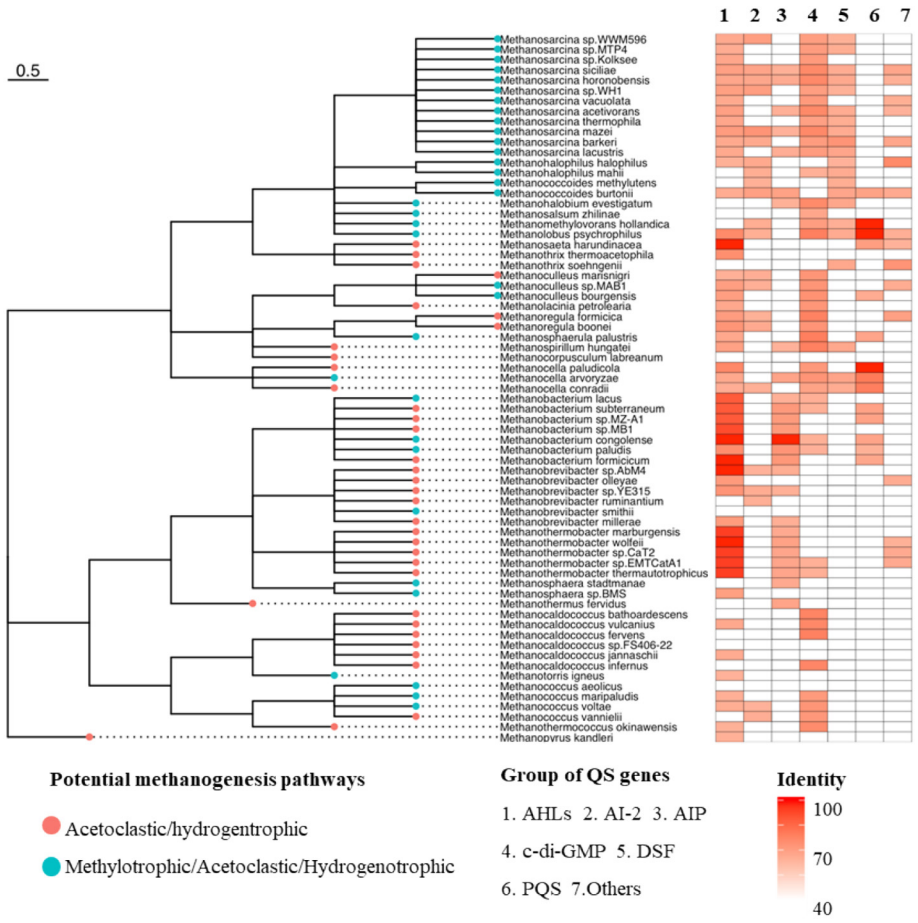

Fig. 2. Potential QS genes in methanogens. Completed genomes of methanogens and the phylogenetic tree was retrieved from NCBI RefSeq database. Scale bar represented 5% sequence divergence. Labels at the tip of the phylogenetic tree represents groups of methanogens based on their methanogenesis pathway. Heatmap shows the presence of QS genes grouped by the signal molecules (AHLs, AI-2, AIP, *c*-di-GMP, DSF, PQS and Others).

wastewater treatment, i.e. *Methanobacterium*, *Methanosaeta* and *Methanosarcina*, all harbor filI/filR. Previous experiments have confirmed that *Methanosaeta harundinacea* used carboxylated AHLs, synthesized by filI, for QS (Li et al., 2015b; Zhang et al., 2012).

In addition to AHL-based QS, other potential QS genes in methanogen genomes were also observed. For example, homologs of *c*-di-GMP synthase (or diguanylate cyclase, DGC) and hydrolase (or phosphodiesterase, PDE) are present in 62.3% (43/69) of the methanogens. Although *c*-di-GMP is well known as the second messenger in bacteria, it has never been reported to be produced by archaea. Instead, these results suggest that the existence of another signal molecule with similar structure to *c*-di-GMP is used by methanogens, most probably *c*-di-AMP (Braun et al., 2019; Romling, 2008). Homologs of lepB (AIP group), and phnA and phnB (PQS group) in *Methanobacterium congolense* and several other methanogens were also found. While validating all these observations will need numerous experimental efforts, this analysis guided us to focus on AHL-based QS systems since they are the most prevalent in methanogen genomes.

3.2. System performance of the investigated methanogenesis systems

During the 83 days operation, the COD concentration and gas production (including methane content) of CON, SEP and CFB are shown in Fig. 3A–B. Within the first 10–20 days of operation (the first stage), the reactors with filters (SEP, CFB) had no obvious advantage over the control (CON) in methane production, probably because the high influent COD caused the accumulation of H^+, as evidenced by the dropped pH (Fig. S2A). In the second to fourth stages, pH remained stable within 7.2– 8.0, and the initial and final effluent COD concentrations of CFB were 590 and 85 mg/L, 744 and 159 mg/L, and 485 and 132 mg/L, respectively. In comparison, the initial and final effluent COD concentrations of SEP were 739 and 111 mg/L, 1102 and 236 mg/L, and 582 and 135 mg/L, while the concentrations of CON were 718 and 108 mg/L, 1225 and 238 mg/L, and 685 and 260 mg/L in the three stages, respectively. The COD degradation capability of CFB was improved by 20.2%, 32.4% and 16.6% compared to SEP, or improved by 17.9%, 39.3% and 29.3% compared to CON. At the end of stage four when the operation reached steady state, the COD removal percentage of CFB, SEP and CON was 96.8%, 96.7% and 93.8%, respectively.

The methane content in the produced gas was similar in these three reactors (Fig. 3B), while the order of the gas production was CFB > SEP > CON, consistent with the trend for the COD removal. The elevated COD removal percentage in CFB and SEP might be attributed to the biofilm formation on filters, which increased the biomass in the system, resulting in promoted COD degradation and methanogenesis. It is also possible that methanogens were enriched in the biofilms. Additionally, the bioreactor with conductive material (CFB) achieved better performance in COD removal compared to SEP and CON bioreactors ($P = 5.86 \times 10^{-4}$ and 1.43×10^{-4}, respectively, by Wilcoxon signed rank test), probably due to the enhanced direct interspecies electron transfer (Yin et al., 2017).

The elevated COD removal percentage in CFB and SEP might be attributed to the biofilm formation on filters, as the formation of biofilms increased the total biomass and thus promoted COD degradation and

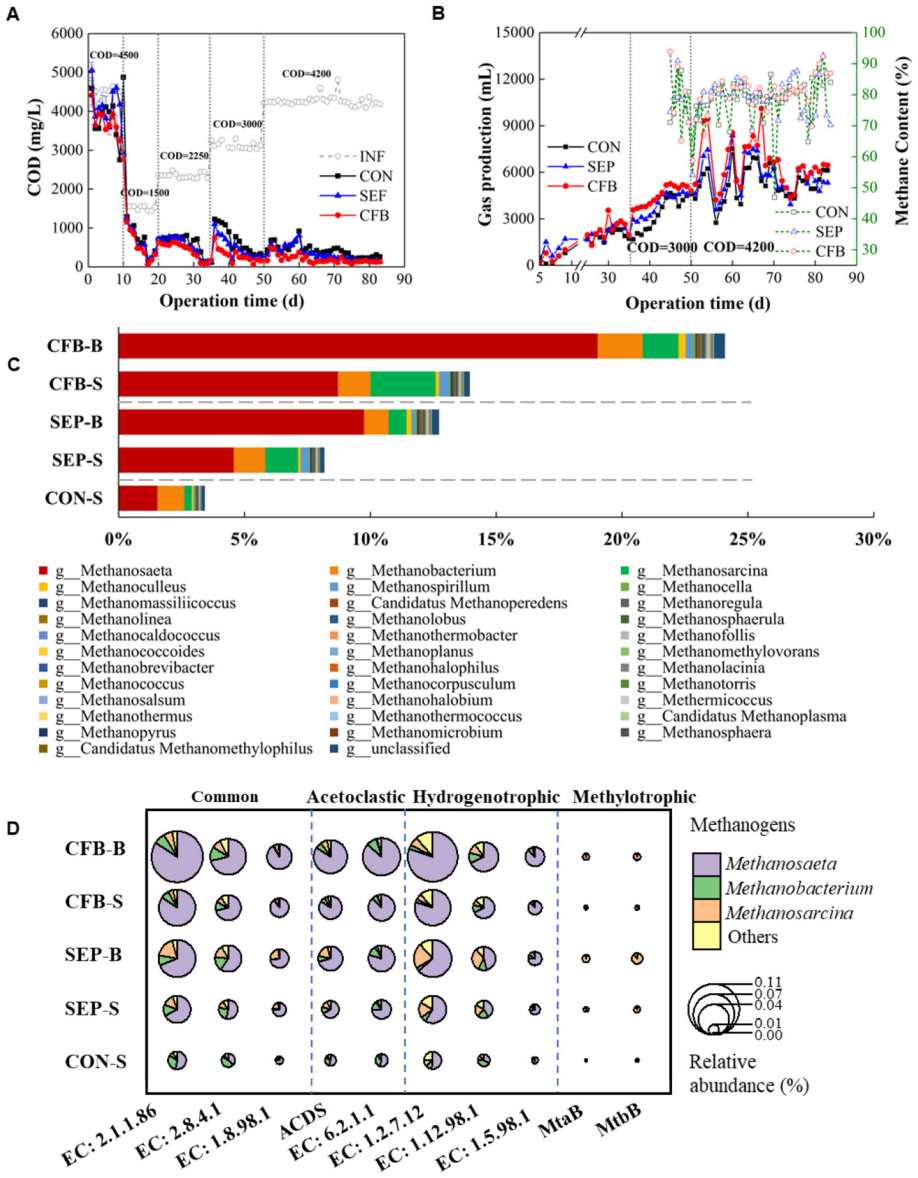

Fig. 3. Performance of the bioreactors during the long-term operation, and the abundance of methanogens. (A) COD concentrations in the influent and effluent during the long-term operation of CON, SEP and CFB; (B) Gas production (left y-axis) and methane content (right y-axis); (C) Relative abundance of methanogens in sludge and biofilm of CON, SEP and CFB; (D) Relative abundance of genes encoding enzymes involved in methanogenesis.

methanogenesis. Additionally, the bioreactor with conductive material (CFB) achieved better performance, probably due to the enhanced direct interspecies electron transfer. Liu et al. (2012) observed higher methane production in the mixture of *G. metallireducens* and *M. barkeri* when active carbon was added to the system, but *G. metallireducens* and *M. barkeri* were not in close contact, suggesting that the activated carbon might help the electron transfer. A study by Rotaru et al. (2014) demonstrated the direct inter-species electron transfer between *M. barkeri* and *G. metallireducens* via conductive pili. Our previous studies also found that the addition of conductive magnetite into bioreactors resulted in improved conductivity and electron transfer (Yin et al., 2018; Liu et al., 2019).

Consistent with the long-term operation, the COD degradation rate was high in CFB and SEP obtained from the batch experiments. Fitting by the modified Gompertz model, the obtained maximum COD degradation rates were 505.4 ± 5.98 mg/L·h in CON, 661.1 ± 5.96 mg/L·h in SEP and 722.2 ± 10.1 mg/L·h in CFB. The batch experiments further validated that biofilm formation could promote COD degradation.

3.3. *Methanogens and methanogenesis genes in the bioreactors*

To investigate methanogens and their functional genes in the three bioreactors, metagenomic sequencing was performed on three suspended biomass (from CON, SEP and CFB, named as CON-S, SEP-S and CFB-S, respectively) and two biofilm biomass (from SEP and CFB, named as SEP-B and CFB-B, respectively) samples. Analysis of the metagenomes in these samples revealed that three most abundant methanogenic genera were *Methanosaeta*, *Methanobacterium* and *Methanosarcina* (Fig. 3C). Their abundances were 1.5%, 1.1% and 0.3% in the suspended biomass of the control bioreactor (CON-S), 4.6%, 1.2% and 1.3% in the suspended biomass of the bioreactor with non-conductive filter (SEP-S), and as high as 8.7%, 1.3% and 2.5% in the suspended biomass of the bioreactor with conductive filter (CFB-S). In the biofilm biomass of the bioreactor with non-conductive filter (SEP-B), the abundances of *Methanosaeta*, *Methanobacterium* and *Methanosarcina* were 9.7%, 1.0%, 0.7%, and were 19.0%, 1.8% and 1.4% of the biofilm of the bioreactor with conductive filter (CFB-B). The

higher abundance of methanogens in the SEP and CFB bioreactors was concordant with the elevated methanogenesis in these reactors, indicating that conductive materials might promote methanogenesis through the enrichment of methanogens.

All these three genera are representative methanogens in anaerobic bioreactors. For example, Gagliano et al. (2017) found that *Methanosaeta harundinacea* was the dominant methanogen in both biofilm and granule. In anaerobic sequencing batch reactors fed with mixed carbon sources, *Methanosarcina*, *Methanosaeta* and *Methanobacterium* dominated (Liu et al., 2019). Yin et al. (2020) found that *Methanosaeta* and *Methanosarcina* were dominant in the vast majority of methanogenic consortia using mixed organic substrates. These suggest that the three genera had strong competitive advantage in anaerobic digesters and biofilms.

Consistent with the abundance of methanogens, the total abundance of genes in methanogenesis pathways was in the order of CFB-B > SEP-B > CFB-S > SEP-S > CON-S (Fig. S2E). Methane is generated from 5-Methyl-tetrahydromethanopterin (THMPT) via enzymes EC:2.1.1.86, 2.8.4.1 and 1.8.98.1, and a step shared between the acetoclastic and the hydrogenotrophic pathways (the Common pathway, Fig. S3). In the acetoclastic methanogenesis pathway (M00357), acetate was transformed to 5-Methyl-THMPT by enzymes EC:6.2.1.21 and ACDS, while in the hydrogenotrophic pathway (M00567), CO_2 was transformed to 5-Methyl-THMPT by enzymes EC:1.2.7.12, EC:1.12.98.1, EC:1.17.1.9, EC:1.5.98.1 and EC:1.5.98.2, and in the Methylotrophic pathway (M00356 and M00563) enzymes such as MtaA, MtaB, MtaC and MtbB generates methyl-CoM from methanol, methylamine and other molecules. Compared to CON, all four pathways were up-regulated in the bioreactor with conductive filter (CFB), but the relative abundance of acetate kinase (EC: 2.7.1) and phosphate acetyltransferase (EC: 2.3.1.8) were down-regulated (Fig. S3). Among the dominant methanogens, *Methanosaeta* primarily utilizes acetate for methane production, while *Methanobacterium* is a typical hydrogenotrophic methanogen, and *Methanosarcina* is able to perform methanogenesis through the methylotrophic pathway (Fig. 3D).

3.4. Quorum sensing genes and molecules

QS genes in the metagenomes of the five samples were analyzed to examine the potential cell-cell communications in the system, and the results are shown in Fig. 4A. The most three abundant QS genes in the systems were filR, fill and lepB. Noticeably, fill and filR both belong to the AHLs group. This group also includes hdtS. When summed up, AHLs were the predominant QS group in these reactors. The lepB gene, a key component of the AIP QS system encoding signal peptidase I, ranked third. AIP was commonly used by Gram positive bacteria for intraspecies communication, and therefore might be carried by syntrophic bacteria and methanogens. DGC and PDE genes, which could synthesize and degrade *c*-di-GMP, respectively, also showed high abundance. Other high abundance QS genes included phnA and phnB of the PQS group, and secDF that was classified in the 'Others' group.

The QS genes were further classified by whether they were carried by methanogens. As shown in Fig. 4B, AHLs were the most abundant QS genes in methanogens in all bioreactors. Their abundance was in the order of CFB-B > SEP-B > CFB-S > SEP-S > CON-S, which was consistent with the abundance of methanogens. QS genes that fall in the 'Others' group were also detected in methanogens. For non-methanogens, the most abundant QS genes belonged to AHLs, *c*-di-GMP and Other groups. Yin et al. (2020) proposed that *c*-di-GMP might be one important signal molecules responsible for the syntrophic interaction in methane producing mixtures. Our observation of abundant *c*-di-GMP related QS genes in methanogenic systems here provided indirect support for this hypothesis. Among the different samples, there were strong variations in abundance of the non-methanogen QS genes and no general trend was observed. However, the driving force of this phenomenon remains to be explored.

The metagenomic data suggested that AHLs might be the primary cell-cell communication system in the bioreactors. To validate this observation, the concentrations of ten signal molecules in the AHL group were measured, namely C_4-HSL, C_6-HSL, C_8-HSL, 3-oxo-C_8-HSL, C_{10}-HSL, 3-oxo-C_{10}-HSL, C_{12}-HSL, 3-oxo-C_{12}-HSL, C_{14}-HSL and 3-oxo-C_{14}-HSL (Fig. 4C–E). These molecules were detectable in the water phase of all samples. No significant difference was observed between the concentrations of AHL concentrations in the water phase of the three bioreactors ($P = 0.85$, Kruskal-Wallis test; Fig. 4C). In the sludge phase, the total concentrations of C_8-, C_{10}- and C_{12}-HSL were much higher in the sludge of SEP and CFB bioreactors compared to that in the sludge of the CON bioreactor ($P = 0.041$ and 0.047, respectively, by *t*-test;

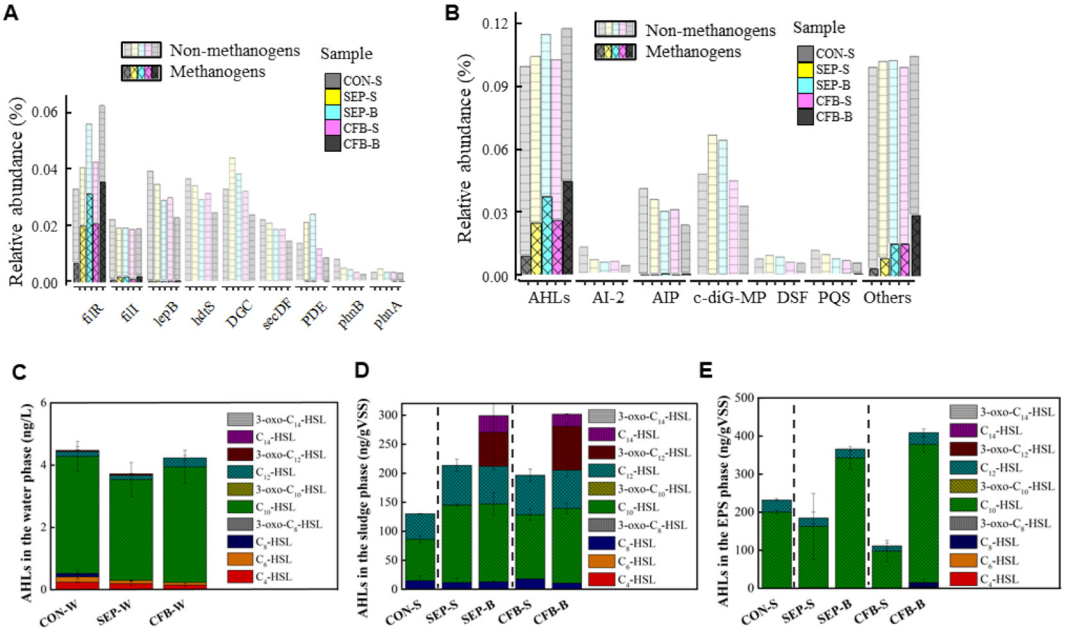

Fig. 4. Distribution of QS genes and signal molecules in the samples. (A) Gene abundance of top ten QS genes in sludge and biofilm from CON, SEP and CFB; (B) cumulative relative abundance of seven groups of QS genes in methanogens and non-methanogens; (C-E) Concentration of AHL signal molecules in the water phase (C), the sludge phase (D), and the EPS phase (E) in sludge and biofilm from CON, SEP and CFB bioreactors, respectively.

Fig. 4D). The concentrations of C_8-, C_{10}- and C_{12}-HSL in the biofilms in CFB and SEP were similar to that in the sludge ($P = 0.98$ and 0.94, respectively, *t*-test). Noticeably, 3-oxo-C_{12}-HSL and C_{14}-HSL were detected in the biofilms of SEP and CFB but not in the sludge. In the extracellular polymeric substances (EPS) phase, the detected AHLs were C_{10}-HSL and C_{12}-HSL (Fig. 4E). Compared to the sludge in CON, the concentrations were lower in the sludge of SEP and CFB, although not statistically significant ($P = 0.69$ and 0.14, respectively, *t*-test) but significantly higher in the biofilms in these two bioreactors ($P = 0.033$ and 0.026, respectively, *t*-test). The detection of abundant AHLs validated the prediction that methanogenesis systems might utilize the AHL system for QS, and the higher concentrations of AHLs in CFB and SEP bioreactors, especially in the biofilms, were consistent with the higher abundance of AHL group QS genes observed by metagenomic sequencing. A significant correlation was observed between the AHL concentrations and the abundance of AHL-group QS genes (Pearson correlation coefficient (r) =0.91 and *p*-value <0.05, Fig. S4).

AHLs are reported to have different solubility and oil-water partition coefficients, therefore their distribution in the environmental medium also differs (Decho, 2015). Short-chain AHLs (C_4-C_8) have good solubility and therefore are mostly distributed in water and EPS phases (Feng et al., 2014), while long-chain AHLs are insoluble and primarily distributed inside the biomass. In our study, the AHLs in the water phase represented the free signal molecules in the bioreactors. In the water phase, the detected AHLs were C_4-HSL and C_6-HSL, which were reported to promote biofilm formation in pure cell cultures (Morgan-Sagastume et al., 2005; Tang et al., 2019a, 2019b; Wagner et al., 2003; Yu et al., 2019).

In UASB reactors, Li et al. (2015b) found that AHLs promoted the growth of *Methanothrix*, and improved sludge granulation and system performance. Hu et al. (2016) also observed that the concentration of AHL was correlated positively with biofilm mass and EPS production. C_4-HSL was found to promote anaerobic sludge granulation (Ding et al., 2015). Lade et al. (2014) showed that C_8-HSL and C_{10}-HSL were produced to regulate biofilm formation on the membrane surface. To advance these findings into engineering practice, Ma et al. (2019) added exogenous C_{10}-HSL and found that C_{10}-HSL at 5000 nM achieved the greatest enrichment of *Methanosaeta*, contributing significantly to anaerobic granule formation. In methanogenesis systems, granulation and biofilm formation could reduce the distance between syntrophic microorganisms, enhance electron transfer and thus improve the methane production of the bioreactors.

3.5. Syntrophic co-occurrence analysis

Methanogenesis is a multistep process requiring a consortium of syntrophic bacteria and archaea. In the five samples from our bioreactors, the nine most abundant genera together represented about 15% to 30% of the microbes (Fig. 5A). Among them, the methanogens *Methanosaeta* and *Methanosarcina* metabolize acetate, while species such as *Geobacter sulfurreducens* and *Geobacter anodireducens* in the *Geobacter* genus, and some species in *Thauera* genus oxidize ethanol to generate acetate. Some species in *Syntrophobacter* and *Smithella* also transform propionate into acetate. Indeed, analysis of the metagenomes showed the presence of functional genes metabolizing ethanol, propionate and acetate. The abundance of genes metabolizing acetate and propionate followed a clear trend of CFB-B > CFB-S > SEP-B > SEP-S > CON-S (Fig. S5A). In addition to the dependence in syntrophy, *Geobacter*, *Anaerolineaceae*, *Ignavibacteriaceae*, *Thauera*, *Syntrophobacter* and *Smithella* all participated in the direct interspecies electron transfer, which might also contribute to methanogenesis (Yin et al., 2020; Zeng et al., 2019).

A correlation network was constructed based on the abundance of the genera in the five samples, in which each node represents one genus, and each edge represents a strong positive correlation (Pearson's

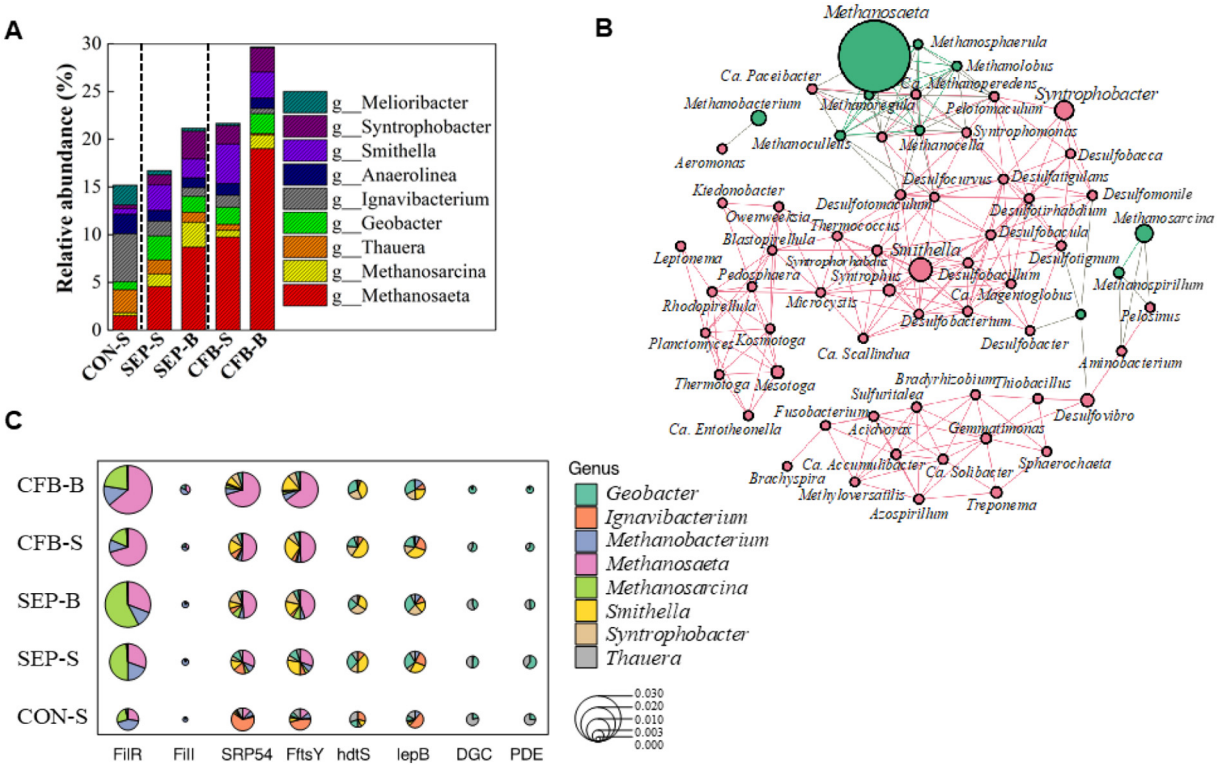

Fig. 5. Correlation between methanogens and the syntrophic bacteria in the methanogenesis systems. (A) Relative abundance of nine most abundant genera in sludge and biofilm from CON, SEP and CFB; (B) Co-occurrence network between syntrophic bacteria and methanogens ($P < 0.05$, Pearson's coefficient > 0.8). Syntrophic bacteria and methanogens are displayed in pink and green nodes, respectively. The size of each dot represents median abundance of each genus; the thickness of each connection is proportional to the Pearson's coefficient. The grey edges show the collaboration between syntrophic bacteria and methanogens; (C) Abundance of the most abundant QS genes in methanogens and their syntrophic bacteria.

correlation coefficient > 0.8 and $P < 0.05$; Fig. 5B). Strong correlation was frequently observed between methanogens and syntrophic bacteria. The known syntrophic relationships were well presented in the network. For instance, *Syntrophobacter*, oxidizing propionate into acetate and CO_2 via the randomizing methylmalonyl-CoA pathway, was a close neighbour of multiple methanogens in the network. *Smithella* transforming propionate into acetate and butyrate, was a hub in the network connecting multiple non-methanogens and indirectly connected with methanogens. The most abundant methanogen, *Methanosaeta*, which is a typical acetoclastic methanogen, directly interacts with *Pelotomaculum* and Ca. *Paceibacter*, and is in the close neighbourhood of *Syntrophobacter* and *Smithella*. Interestingly, *Methanosarcina*, which is able to produce methane using all three methanogenesis pathways, is located in a different module in the network and directly links with *Aminobacterium* and *Pelosinus* but has no direct link with *Syntrophobacter* or *Smithella*. These observations demonstrate that the correlation network reflects some known syntrophic relationship and may provide clue for potential new syntrophic interactions. However, it should be noted that correlation is not identical to causal relationship. Experimental evidence will be needed to confirm a new syntrophic connection.

The QS genes that were carried by methanogens and their syntrophic partners are shown in Fig. 5C. The filI/filR system was carried by methanogens, while DGC and PDEs were specific to the non-methanogens including *Geobacter* and *Thauera*. hdtS, a member of AHL, and lepB, a member of AIP, were carried by both methanogens and *Smithella*. Therefore, the AHL and AIP systems might be used by methanogens and the syntrophic bacteria to perform inter-species communication.

4. Conclusions

QSDB was constructed to search for potential QS genes in methanogens. Mining the genomes of methanogens indicated that AHLs and AIPs might be major QS pathways used by methanogens. In anaerobic digestion bioreactors, conductive material enhanced methane production through the enrichment of methanogens. QS genes carried by methanogens in the anaerobic digestion bioreactors were primarily assigned to the AHL system, and the AHL signal molecules were experimentally detected. The co-occurrence network revealed well-known syntrophic bacteria such as *Smithella* and *Syntrophobacter*, and they were predicted to carry DSF and *c*-di-GMP genes for cell-cell communication. AIP genes were harbored by both methanogens and their syntrophic bacteria, which might be responsible for interspecies communication. Overall, this study provides helpful resources for QS studies, and the results also improve our understanding of QS in anaerobic digestion systems. Future work can focus on applying the findings in engineering practice to regulate methane production. Future work can also make use of the constructed database to predict potential QS systems in unknown microorganisms especially in anaerobic microbes and archaea. Meanwhile, experimental evidence is important to validate the predictions, and the validated findings will in turn be integrated to improve the database.

CRediT authorship contribution statement

Qing Du: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **Quanhua Mu:** Formal analysis, Writing – review & editing. **Guangxue Wu:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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