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Quorum sensing between Gram-negative bacteria responsible for methane production in a complex waste sewage sludge consortium

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Abstract

Quorum sensing (QS) plays a key role in activating bacterial functions through small molecules called autoinducers. In this study, the QS of Gram-negative bacteria in waste sewage sludge (WSS) was downregulated by adding the quorum quenching enzyme, AiiM lactonase, which cleaved the acyl-homoserine lactone (AHL) autoinducer signals from Gram-negative bacteria, and subsequently methane production was inhibited by over 400%. The pH was lowered after 2 days in the anaerobic fermentation whereas protease activity at the hydrolysis step was almost the same with or without AiiM. The production of acetic acid significantly increased during the fermentation in the presence of AiiM. The bacterial community at day 2 indicated that the population of Gram-positive bacteria increased in the presence of AiiM, and the percentage of Gram-negative bacteria decreased in the WSS containing AiiM. The change in the bacterial community in the presence of AiiM may be due to the different antimicrobial agents produced in the WSS because some of the Gram-positive bacteria were killed by adding the solid-phase extraction (SPE) fraction from the WSS without AiiM. In contrast, the SPE fraction with AiiM had reduced bactericidal activity against Gram-negative bacteria. Thus, bacterial signaling between Gram-negative bacteria is critical for methane production by the microbial consortia.

Keywords Anaerobic digestion · Waste sewage sludge · Quorum sensing · AHL lactonase · Gram-negative bacteria

Introduction

Quorum sensing (QS) is an important system for regulating bacterial gene expression through the use of signal molecules such as acyl-homoserine lactones (AHL), autoinducer-2, cholera autoinducer (CAI-1), and indole for Gram-negative bacteria (Galloway et al. 2011; Lee et al. 2015) and autoinducer oligopeptides for Gram-positive bacteria (Sturme et al. 2002). These signal molecules, known as autoinducers, regulate several functions of bacterial communities including biofilm formation, plasmid conjugal transfer, and the production of virulence factors (Huang et al. 2016) which inhibit other bacteria, fungi, protozoa, and nematodes (Burgess et al. 1999; Dubuis et al. 2007). For Gram-negative bacteria, the general method to inhibit bacterial QS is known as quorum quenching (QQ). QQ inhibits QS through several ways: by degrading autoinducers enzymatically (e.g., AHL lactonase and AHL acylase), blocking the production of autoinducers, and blocking the interaction of autoinducers with a receptor protein (Dong et al. 2000; Guendouze et al. 2017; Mayer et al. 2015). Among the QQ enzymes, AHL lactonases disrupt QS by cleaving the lactone ring of the AHL. For example, the AHL lactonase encoded by *aiiM* from *Microbacterium testaceum* StLB037 inhibits the QS of the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (Wang et al. 2010). In addition, when *aiiM* was cloned into

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Pseudomonas aeruginosa PAO1, AiiM reduced the production of AHL-mediated virulence factors and reduced cytotoxicity in human lung epithelial cells (Migiyama et al. 2013). To date, AHL lactonases are well-studied for understanding the effect of QS inhibition for monoculture and dual-culture bacterial systems (Cheong et al. 2013; Guendouze et al. 2017; Kim et al. 2011; Tan et al. 2015).

Biological wastewater treatments produce a large amount of waste sewage sludge (WSS) which contains a large population of microbes that exist in biofilms, flocs, and granules (Mikkelsen and Keiding 2002; Oh et al. 2013). These microbial consortia, consisting of a large number of microbes including Gram-positive and Gram-negative bacteria, degrade a wide range of waste compounds (Dhall et al. 2012; El-Bestawy et al. 2005), denitrify nitrogen sources in the wastewater (Zielińska et al. 2016), and remove phosphorus compounds (Ivanov et al. 2005). There are a variety of AHL molecules in microbial consortia (Tan et al. 2015), and the AHLs may regulate the activity of Gram-negative bacteria (Huang et al. 2016). Some of the recent studies focus on the relationship between QS bacteria and QQ bacteria as well as the effect of QQ enzymes on the microbial communities in biological wastewater treatment processes; for example, the addition of QQ bacteria influences QS (Cheong et al. 2013; Oh et al. 2013). Cheong and co-workers used *Pseudomonas* sp. 1A1, a QQ bacterium, to control biofouling in a lab-scale membrane bioreactor (MBR) for wastewater treatment. This system reduced membrane biofouling through the QQ acylase enzyme produced by *Pseudomonas* sp. 1A1 (Cheong et al. 2013). When investigating the QQ mechanisms of *Rhodococcus* sp. BH4, particularly in a wastewater treatment MBR, Oh and co-workers showed that strain BH4 can degrade a wide range of AHLs and that the QQ activity of *Rhodococcus* sp. BH4 in batch reactors coincides well with biofouling inhibition found in a continuous MBR (Oh et al. 2013). Also, the immobilization of an AHL acylase on nanofiltration membranes reduced biofouling in wastewater treatment by inactivating the QS of microorganisms in the membrane biocake (Kim et al. 2011). Jiang and co-workers also found QQ controls biofouling by reducing the sludge particle size, the apparent viscosity, and the biofilm strength (Jiang et al. 2013). In addition, Tan and co-workers investigated the relationship between QS and QQ activities using floccular and granular sludge and found that the floccular sludge has high QQ activity and that the QQ activity of the community reduced when the floccular biomass is transformed into granular sludge (Tan et al. 2015). The above studies indicate that the balance between QS and QQ influences the performance of each bacterial fermentation (Cheong et al. 2013; Mayer et al. 2015; Oh et al. 2013; Tan et al. 2015).

Anaerobic digestion of WSS is one of the important approaches to reduce the amount of WSS by producing methane. There are four stages in the anaerobic digestion of WSS: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the hydrolysis stage, large molecules (e.g., protein, carbohydrate, and lipid) are converted into amino acids, sugars, and fatty acids by extracellular enzymes such as proteases, amylases, cellulases, and lipases which are produced by hydrolytic bacteria. In the acidogenesis and acetogenesis stages, acidogenic bacteria convert the small molecules into alcohols, volatile fatty acids (e.g., acetate, propionate, and butyrate), hydrogen, and carbon dioxide; thereby, acetate and hydrogen can be utilized by methanogenic archaea and bacteria in the methanogenesis step (Appels et al. 2008; Ziemiński and Frąc 2012). Thus, a complex microbial community in WSS is essential for methane production.

Recently, the effect of antibiotics such as azithromycin and chloramphenicol on methane fermentation was studied (Mustapha et al. 2016; Nguyen et al. 2014). Moreover, a key microorganism as an accelerator or a suppressor in the methane fermentation was determined by comparing active bacterial communities in each WSS sample (Mustapha et al. 2018; Mustapha et al. 2017). These results imply a complicated bacterial interaction and indicate the need to understand the bacterial network for enhanced methane production. Among the microbes in WSS, bacteria may have a key role in the degradation of WSS and consist mainly of Gram-negative and Gram-positive bacteria. Since WSS occurs in bacterial aggregates, QS systems of Gram-negative and Gram-positive bacteria should be active. For the Gram-negative bacteria, AHL signals should be used for activating various bacterial functions. Similarly, Gram-positive bacteria should regulate bacterial virulence and activity via oligopeptides. Hence, we reasoned that the interaction between Gram-negative and Gram-positive bacteria may be important for methane production during the anaerobic digestion of WSS. Therefore, the motivation of this study was to understand the balance between Gram-negative and Gram-positive bacteria during the methane fermentation. Hence, we studied the effect of AHL degradation by adding AHL lactonase, AiiM, during the methane fermentation. For this approach, AiiM derived from *M. testaceum* StLB037 (Wang et al. 2010) was cloned, purified, and utilized to evaluate methane fermentation using WSS. Our results demonstrate that AiiM lactonase inhibits methane production significantly by changing the production of antimicrobial molecules that inhibit Gram-negative and Gram-positive bacteria.

Materials and methods

Waste sewage sludge and chemicals

WSS was routinely obtained from the Hiagari Wastewater Treatment Plant in Kitakyushu City, Japan. WSS was washed

using distilled water and centrifuged as described previously (Mustapha et al. 2018). Then, the final concentration of WSS was adjusted with distilled water to 10% (*w*/*w*). C_6-HSL, C_8-HSL, 3-oxo-C_6-HSL, and 3-oxo-C_8-HSL were purchased from Sigma-Aldrich (St. Louis, MO). All of these substrates were dissolved and prepared in methanol at 100 μM. Bovine serum albumin (BSA) was purchased from New England Biolabs (Japan). A peptidase enzyme (carboxypeptidase Y) was purchased from Nacalai Tesque (Japan).

Bacterial strains and growth conditions

All the bacteria used in this study were first cultured on Luria-Bertani (LB) agar with or without an appropriate antibiotic. *Escherichia coli* strain M15/pREP4 was cultured with 30 μg/mL carbenicillin at 37 °C and was used as a host for expressing an AHL lactonase enzyme. The two AHL biosensors, *Chromobacterium violaceum* CV026 (50 μg/mL kanamycin) and *Agrobacterium tumefaciens* NTL4 PZLR4-traG:lacZ (30 μg/mL gentamycin), were grown at 30 °C with 250 rpm. *P. aeruginosa* PA14 was cultured at 37 °C and was used to check QQ by an AHL lactonase. *Bacillus subtilis* 168, *Brevibacillus* sp. KH3, and *Lactococcus lactis* 12007 were used as representative Gram-positive bacteria, and *C. violaceum* CV026, *Salmonella typhi* 14028, and *A. tumefaciens* NTL4 PZLR4-traG:lacZ were used as representative Gram-negative bacteria to check the bactericidal effects of the antimicrobial molecules produced by the WSS with or without an AHL lactonase. Solid bacterial media were made by adding agar at a final concentration of 1.5% to the liquid media. *Methaosarcina acetivorans* C2A was used to evaluate the effect of AiiM on its growth and methane production; this strain was cultured in HSYE-methanol medium as previously reported (McAnulty et al. 2017).

Methane production at the different concentrations of AiiM

Gene cloning (*aiiM*, accession number AB513359), protein purification, and confirmation of QQ activity by AiiM are described in the supplementary file. WSS (30 mL) containing 0 (control), 0.6, 1.2, or 2.4 μg/mL of AiiM was prepared in a 66-mL vial. Each vial was tightly sealed with a rubber stopper and aluminum cap and sparged with nitrogen gas for 2 min to create an anaerobic atmosphere. The vial was then incubated at 37 °C with 120 rpm for 10 days. Each experiment was conducted at least in triplicate. The amount of methane was measured by a GC-3200 gas chromatograph (GL Sciences, Japan) by injecting 100 μL of headspace gas of each vial during the fermentation for 10 days as previously described (Mohd Yasin et al. 2015). BSA (2.4 μg/mL) and peptidase (2.4 μg/mL) were added to the WSS to check the effects of protein (BSA) and peptide signal degradation (peptidase) on methane production. Purification buffer (300 μL) with 0.05 mM PMSF was also added to 30 mL WSS to evaluate the effect of dilution on methane production.

Other analytical methods

Each sample was centrifuged at 13,000 rpm for 7 min to collect the supernatant and was further filtered with a 0.2-μm pore membrane syringe filter. The supernatant was utilized to measure organic acids by high-performance liquid chromatography (Shimadzu LC-10AD) as previously described (Mohd Yusoff et al. 2012) and pH by a compact pH meter (AS ONE, AS-211, Japan). In addition, the soluble protein concentration was measured by the Lowry method using bovine serum albumin (BSA) as a standard protein (Lowry et al. 1951). Protease activity was measured as described previously (Maeda et al. 2011). One unit of protease activity was calculated as the quantity of tyrosine (μmol) produced from casein by 1 mg of enzyme per minute. Each experiment was conducted at least in triplicate. In addition, Gram staining was performed (Supporting Information) by using the samples of the WSS control and WSS-AiiM which were incubated anaerobically for 2 days (as described in the methane production preparation).

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis from the pellets of the control WSS and the WSS with AiiM were performed as described previously (Mustapha et al. 2017). The total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), and the total RNA concentration was measured by using the NanoDrop ND-1000 spectrophotometer (SCRUM Inc., Japan). The complementary deoxyribonucleic acid (cDNA) was synthesized using the PrimeScript RT Reagent Kits (TAKARA Bio Inc., Shiga, Japan) and was performed as described previously (Mustapha et al. 2017). The cDNA was then used as a template to evaluate microbial communities.

High-throughput 16S rRNA sequencing and data processing

16S rRNA genes were amplified using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') targeting the V3 and V4 regions (Klindworth et al. 2013). All steps were processed according to the Illumina protocol for preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system. Detailed procedures are described in our previous paper (Mustapha et al. 2018). The data obtained were demultiplexed and the reads were then classified to different taxonomic levels. The raw sequence data were deposited in the National Center for Biotechnology Information (NCBI) short reads archive database (accession number,

SRP072534). The acquired data were processed as previously described (Mustapha et al. 2018) (Supporting Information)

Gram staining

Gram staining was performed by using the samples of the WSS control and the WSS-AiiM sample which were incubated anaerobically for 2 days (as described in the methane production section). After 2 days, 2-mL samples were taken and filtered with a 5-μm pore size filter paper (Toyo Roshi Kaisha, Ltd, Japan) to collect the microbial cells. The filtered samples were used for the Gram staining. First, 10 μL of each sample was transferred onto a glass slide and gently spread using a pipette tip; the sample was fixed on the glass slide by heating and treated using a a Gram Stain kit (BD, USA). The final samples were observed at a × 100 magnification by a microscope (Olympus, BX51, Japan), and the number of Gram-positive bacteria and Gram-negative bacteria was determined (5 random positions were chosen).

Bactericidal test

Organic compounds from WSS samples with or without the addition of AiiM were extracted using the method of solid-phase extraction (SPE) as previously described (Chau et al. 2017) with a slight modification (Supporting Information).

The SPE solutions were used for a disk-agar diffusion method to evaluate the bactericidal effect. Briefly, the overnight culture of bacteria used in this assay (100 μL) was inoculated into 100 mL of LB agar medium, heated at 45 °C, and immediately poured and solidified into a petri dish (20 mL). Then, a filter paper (8-mm diameter) was put on the surface of each agar plate and 20 μL of each SPE solution was added to the filter paper. The agar plates were incubated for 24 h to visually evaluate the bactericidal effect by seeing a zone of growth inhibition.

For the quantitative assay of the bactericidal effect by the SPE solutions, the survival of tester strains was evaluated (see supplementary materials and methods in the supplementary file).

Statistical analysis

Means were calculated from at least triplicate data ($n = 3$). Comparisons were performed using means and standard deviations by Student's t test (GraphPad software) at a significance level of $p < 0.05$.

Results

Effect of AiiM lactonase on methane production

Three concentrations of AiiM (0.6, 1.2, and 2.4 μg/mL) were used to see the effect of AiiM on methane production. As a result, methane production using WSS was significantly inhibited in the presence of AiiM (Fig. 1). Higher concentrations of AiiM showed higher inhibitory effects on methane production. The protease inhibitor, PMSF, which was initially used during the AiiM purification step, was also tested on methane production. PMSF at a concentration of 0.5 mM was found to have no effect on methane production (see Fig. S4a).

Additionally, BSA (negative control) and peptidase were also added to WSS at the same concentration as AiiM (2.4 μg/mL) to see the effect of autoinducer oligopeptides on the methane fermentation. There was no difference in methane production using BSA or peptidase compared to the control WSS (see Fig. S4b). Taken together, methane production using WSS was inhibited by the AiiM lactonase.

Bacterial activities in the presence of AiiM lactonase in methane fermentation

To figure out the mechanism by which AiiM reduced methane production in WSS, each stage of the methane fermentation (hydrolysis, acidogenesis/acetogenesis, and methanogenesis) was investigated. As the first step, the soluble protein concentration and protease activity were measured after 2 and 4 days of fermentation to compare the hydrolysis step with or without AiiM; however, there was no significant difference with or without AiiM although a slightly high protease activity was detected (Fig. S5). Thus, the hydrolysis process was not affected by the addition of AiiM.

As a second step, the production of organic acids and pH were measured during the 10 days of the fermentation to compare acidogenesis/acetogenesis with or without AiiM. The pH was drastically reduced for WSS with all concentrations of AiiM addition after 2 days. The reduction occurred in a dose-dependent manner as the initial pH value changed to

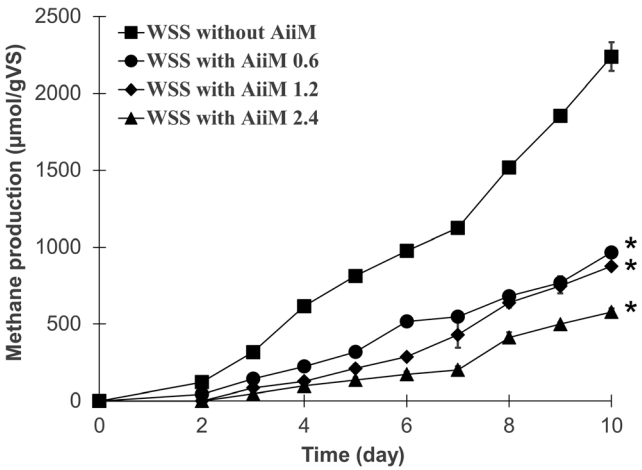

Fig. 1 Impact of AiiM AHL lactonase on methane fermentation using waste sewage sludge (WSS). WSS without AiiM (*dark squares*), AiiM at 0.6 μg/mL (*dark circles*), at 1.2 μg/mL (*dark diamonds*), and at 2.4 μg/mL (*dark triangles*) is shown. Error bars indicate standard errors ($n = 3$). Asterisk indicates a significant difference in the presence of AiiM

5.60 ± 0.07 (0.6 μg/mL AiiM), 5.20 ± 0.00 (1.2 μg/mL AiiM), and 4.80 ± 0.00 (2.4 μg/mL AiiM) as shown in Fig. 2a. During the fermentation, the propionic acid, acetic acid, and formic acid were detected in all samples with or without AiiM. However, the amount of each organic acid produced in each sample was different. A higher concentration of propionic acid was detected in the control WSS compared to WSS with the addition of AiiM from 2 to 10 days of fermentation (Fig. 2b). In contrast, the concentrations of acetic acid and formic acid were higher in the WSS with AiiM than the WSS control (Fig. 2c, d). In particular, the amount of acetic acid after 2 days in WSS-AiiM was significantly higher than the WSS control. Also, the concentration of formic acid in the WSS control was initially higher and was quickly consumed at the end of the incubation period. However, its concentration in the WSS with AiiM remained high during the fermentation. This higher production of organic acids, particularly acetic acid and formic acid, caused the large reduction in pH for the WSS with AiiM.

As the last step, the methanogenesis stage was evaluated by two approaches: the first one measured the production of methane after controlling pH for 2 days at pH 7.0 (the methodology was presented in Supporting Information) and the second one tested the effect of AiiM on *M. acetivorans* C2A, a versatile methane-producing microbe (the methodology was presented in Supporting Information). As a result, methane production was restored by the pH control to the same amount of methane as the control and AiiM had no impact on growth or methane production by *M. acetivorans* C2A. Therefore, the reason why less methane was found in the presence of AiiM was due to the difference in production of organic acids, in particular, due to the lower pH with AiiM.

Richness and diversity of microbial communities in the presence of AiiM lactonase

The comparison of richness and diversity in the microbial communities was performed using control WSS and WSS with the highest concentration of AiiM (2.4 μg/mL). The operational taxonomic units (OTUs) and the Chao1 index were used to determine the richness of the bacterial community. The data in Table 1 reveal the OTUs and the Chao1 value in the WSS control were higher than those in the WSS with AiiM at both 2 days and 10 days. In addition, the Shannon index, which estimates the diversity of the bacterial population, showed a reduction trend during the anaerobic fermentation for both WSS control and WSS with AiiM compared to the original WSS (0 day). This indicates that the fermentation process, which consists of different stages, affected the bacterial diversity and that the addition of AiiM slightly influenced the diversity compared to the WSS control.

Dynamics of the bacterial population and Gram staining in the presence of AiiM lactonase

RNA templates of the WSS control and WSS-AiiM (2.4 μg/mL) were analyzed after 2 and 10 days to compare the taxonomy levels and the dynamics as well as the proportion of Gram-negative and Gram-positive bacteria in the bacterial community.

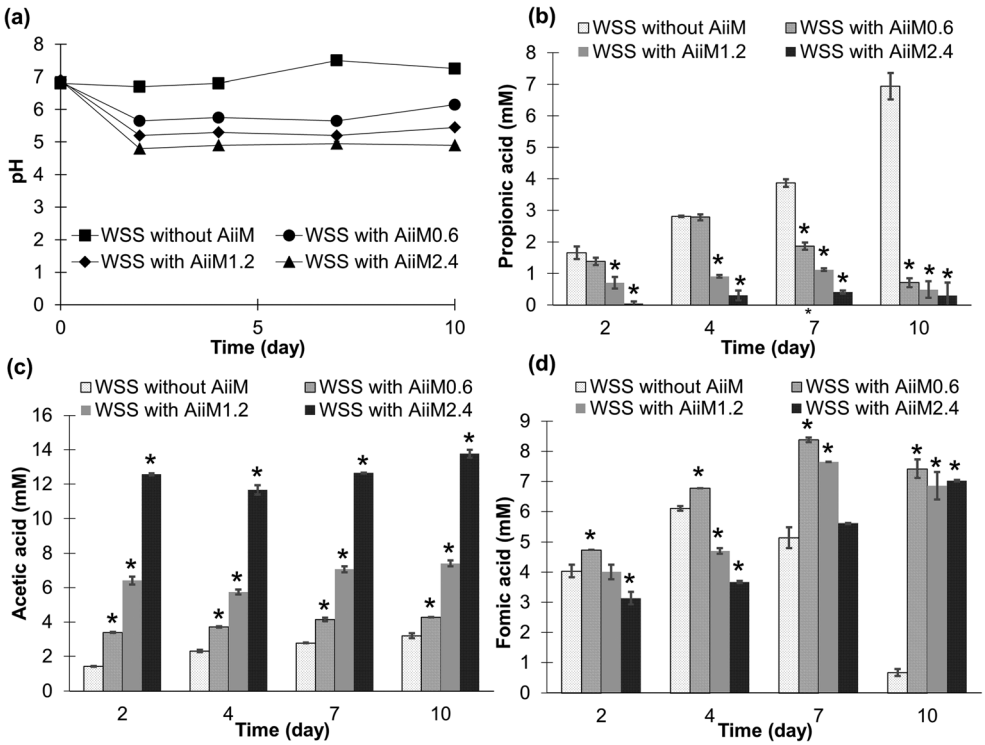

Fig. 2 pH values and organic acids produced during the methane fermentation using WSS with or without AiiM. In the fermentation process, the pH value (**a**) and organic acids including propionic acid (**b**), acetic acid (**c**), and formic acid (**d**) were monitored. Error bars indicate standard errors ($n = 3$). Asterisk indicates a significant difference in the presence of AiiM

Table 1 Operational taxonomic units (OTUs) and alpha diversity (Chao1 and Shannon index) of the bacterial community in WSS with and without AiiM (2.4 μg/mL)

		OTUs[a]	Chao1[a]	Shannon index[a]
WSS	0 day	1779.1	2223.9	3.548
	2 days	1794.8	2231.9	3.452
	10 days	1849.7	2183.3	3.014
WSS-AiiM	2 days	1688.7	1975.8	3.515
	10 days	1714.7	1985.6	3.203

[a] Values were defined using a dissimilarity level of 0.03

The composition of the bacterial community was determined at the phylum level, and *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Plantomycetes*, *Nitrospirae*, *Spirochaetes*, and *Actinobacteria* were the seven dominant phyla of the 13 phyla analyzed (Table 2, Fig. S6, Supporting Information). After the second day, the percentages of three phyla *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* in WSS-AiiM were higher than those in the control WSS. In particular, the *Firmicutes* phylum increased quickly in the presence of AiiM for the WSS community (16 ± 7% in WSS-AiiM and 4 ± 2% in WSS, Table 2). Meanwhile, the other ten phyla showed lower percentages in the WSS-AiiM community than those in the control WSS. Of the 13 total phyla identified in the WSS and WSS-AiiM communities, two Gram-positive phyla (*Firmicutes* and *Actinobacteria*) were present at higher percentages in the WSS with AiiM than those in the control WSS (Table 2) although there was a slight reduction of the *Actinobacteria* percentage in WSS with AiiM after 10 days. On the other hand, the remaining 11 phyla belonged to Gram-negative bacteria. As corroborating evidence that there were more Gram-positive bacteria in the WSS with AiiM, Gram staining was conducted using two samples with or without AiiM after 2 days. As shown in Fig. 3, a significantly large number of Gram-positive bacteria were observed in the WSS with AiiM compared to the WSS control. Meanwhile, a lower number of Gram-negative bacteria were observed in WSS with AiiM.

Dynamics of antimicrobial activities with AiiM lactonase

Based on the observations that AiiM lactonase itself does not have any bactericidal effect and that the addition of AiiM lactonase influences bacterial dynamics when compared to the control, it was hypothesized that the production of antimicrobial molecules may be different in the WSS sample with AiiM lactonase because AHL-based QS system can be inactivated by the enzyme. Therefore, antimicrobial molecules (organic compounds) in each WSS sample (with or without AiiM) were extracted by the SPE method. The quantitative bactericidal assay using *C. violaceum* CV026 (as a representative of Gram-negative bacterial strain; McClean et al. 1997) and *B. subtilis* 168 (as a representative of Gram-positive bacterial strain; Borriss et al. 2017) was tested by counting the number of colonies after the treatment by SPE-WSS-control or SPE-WSS-AiiM for 30 h (Fig. 4a, b). Interestingly, the survival of *C. violaceum* CV026 was reduced by the treatment using SPE-WSS-AiiM (1% *v*/*v*) whereas no bactericidal effect was observed using SPE-WSS-control at the same concentration (Fig. 4a). On the other hand, as shown in Fig. 4b, the survival of *B. subtilis* 168

Table 2 The percentage of Gram-negative and Gram-positive phyla in WSS and WSS-AiiM (2.4 μg/mL) after 2 days and 10 days of anaerobic incubation. The data represent the mean ± SD

Phylum	Percentage of phylum abundance (%)				
	WSS-2d	WSS-10d	WSS-AiiM-2d	WSS-AiiM-10d	Gram staining
Proteobacteria	44 ± 8	34 ± 4	37 ± 8	32 ± 9	Gram-negative
Bacteroidetes	17 ± 3	21 ± 3	21 ± 1	25 ± 4	Gram-negative
Planctomycetes	7 ± 3	3 ± 2	4.8 ± 0.3	3 ± 1	Gram-negative
Nitrospirae	6.9 ± 0.7	1.05 ± 0.07	6 ± 3	0.9 ± 0.6	Gram-negative
Chloroflexi	5.8 ± 0.6	6.0 ± 0.2	4.7 ± 0.8	6.0 ± 0.4	Gram-negative*
Spirochaetes	4.2 ± 0.3	13.1 ± 0.6	1.4 ± 0.4	4 ± 2	Gram-negative
Verrucomicrobia	3.1 ± 0.8	1.3 ± 0.4	2.5 ± 0.2	0.9 ± 0.3	Gram-negative
Acidobacteria	1.0 ± 0.0	0.2 ± 0.0	0.9 ± 0.2	0.45 ± 0.07	Gram-negative
Elusimicrobia	0.5 ± 0.0	0.4 ± 0.2	0.35 ± 0.07	0.3 ± 0.0	Gram-negative
Cyanobacteria	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	Gram-negative*
Chlorobi	0.4 ± 0.1	1.4 ± 0.7	0.5 ± 0.4	0.9 ± 0.6	Gram-negative
Firmicutes	4 ± 2	5.5 ± 0.0	16 ± 7	17 ± 7	Gram-positive**
Actinobacteria	1.7 ± 0.4	6.3 ± 0.9	1.9 ± 0.7	5 ± 1	Gram-positive
Total Gram-negative	89.9 ± 0.3	82.1 ± 0.0	79 ± 8	74 ± 8	
Total Gram-positive	6.0 ± 0.2	11.8 ± 0.9	17.6 ± 0.7	22 ± 8	

*Mostly Gram-negative; **only the *Veillonellaceae* family is Gram-negative

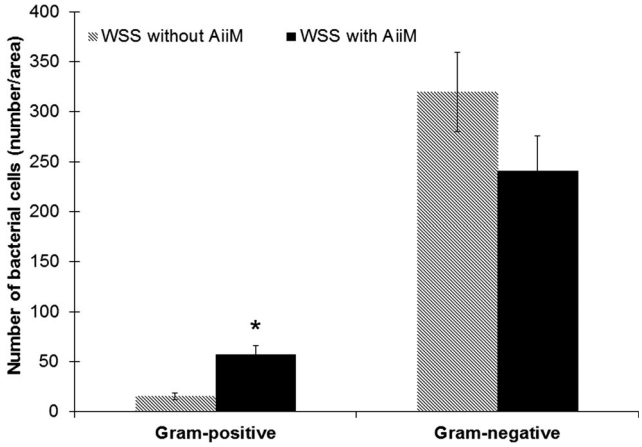

Fig. 3 The number of Gram-negative and Gram-positive bacteria in WSS with and without AiiM. The concentration of AiiM was 2.4 μg/mL. Samples were taken after 2 days of incubation. Error bars indicate standard errors ($n = 3$). Asterisk indicates a significant difference in Gram-positive bacteria after the addition of AiiM

decreased quickly by 1% SPE-WSS-control within 2 h. In contrast, 1% of SPE-AiiM did not influence the growth of *B. subtilis* 168. In the addition, each SPE fraction was tested for a bactericidal effect on the other representative Gram-negative and Gram-positive strains; *B. subtilis* 168, *Brevibacillus* sp. KH3 (Maeda et al. 2011), and *L. lactis* 12007 (Kato et al. 2001) as Gram-positive bacteria and *C. violaceum* CV026, *S. typhi* 14028 (Jarvik et al. 2010), and *A. tumefaciens* NTL4 PZLR4-traG:lacZ (Luo et al. 2003) as Gram-negative bacteria. We found the SPE fraction from the WSS without AiiM showed a remarkable ability to inhibit the growth of all three Gram-positive strains tested in this study (Fig. S7a, S7b, S7c), but it did not affect the survival of Gram-negative strains tested (Fig. S7d, S7e, S7f). In contrast, the SPE fractions from the WSS with AiiM prevented the growth of Gram-negative bacteria but did not have any impact to the Gram-positive bacteria. Note that the methanol was used as a solvent to elute SPE fractions did not affect bacterial growth as no inhibition halo was observed (data not shown).

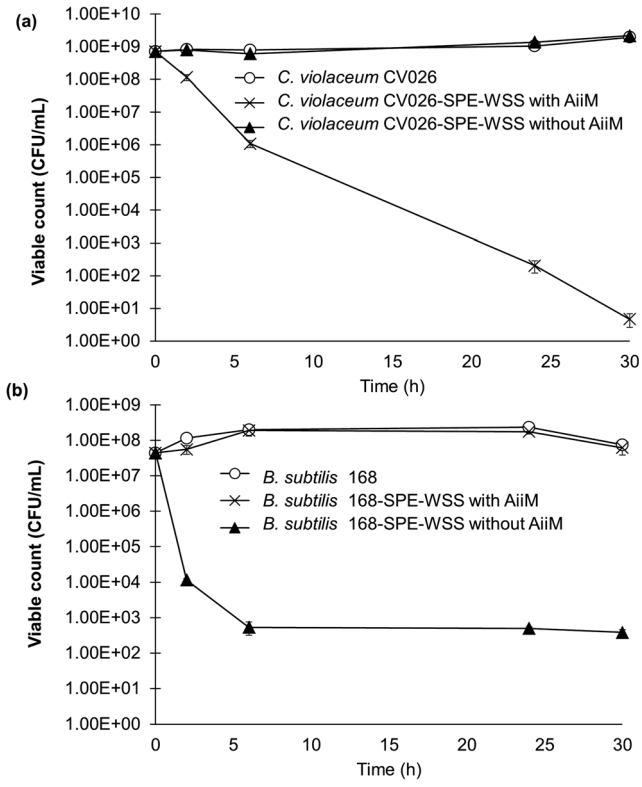

Fig. 4 Inhibition by SPE solutions produced from WSS with and without AiiM. The survival of *B. subtilis* 168 (**a**) and *C. violaceum* CV026 (**b**) with SPE-WSS (1% *v*/*v*) and SPE-WSS-AiiM (1% *v*/*v*). Error bars indicate standard errors ($n = 3$)

Discussion

In this study, the effect of AHL-based QS of Gram-negative bacteria on methane fermentation was investigated by exogenous addition of an AHL lactonase, which is an enzyme that catalyzes the cleavage of the lactone ring in AHLs; thereby, AHL-based QS of the Gram-negative bacteria was inactivated. WSS is a microbial consortium containing not only bacteria (Gram-negative and Gram-positive) but also fungi, virus, and protozoa (Gerardi 2006); therefore, anaerobic digestion of WSS to produce methane is a complex process with many microbial interactions. On the other hand, there is one report that Gram-negative bacteria are dominant in the biological wastewater treatments including the anaerobic digestion process (Huang et al. 2016); hence, the attempt to inactivate the system of QS in the Gram-negative bacteria is a reasonable approach to influence methane production. Although there are many types of QS systems in Gram-negative bacteria such as AHL, AI-2, PQS, indole, and CAI-1 (Galloway et al. 2011; Rutherford and Bassler 2012), only a limited approach is available to inactivate the QS system of Gram-negative bacteria; in particular, only the QQ method for inactivating AHL-based QS has been studied. For example, C-30 brominated furanone interrupts QS by interacting with transcriptional regulator protein LuxR (Maeda et al. 2012), cyclodextrin (CD) creates AHL-CD inclusion complexes thereby inhibiting QS (Morohoshi et al. 2013), and four kinds of enzymes (lactonase, acylase, reductase, cytochrome oxidases) can degrade or modify AHLs (Grandclément et al. 2015). In this study, an AHL lactonase (AiiM) was used as a QQ enzyme to evaluate the impact of QS on methane production. As a result, we found that methane production using WSS is inhibited by AHL lactonase (Fig. 1); hence, AHL signaling increases methane production.

In understanding the mechanism by which AHL signaling increases methane production, we analyzed the methane-producing pathway and the bacterial communities. Based on

that, we found the following results: (1) the hydrolysis process of WSS was not influenced by the AHL lactonase; (2) pH reduction occurred after fermentation with the AHL lactonase (Fig. 2a); (3) the profile of organic acids produced during the fermentation was different with the AHL lactonase; in particular, acetic acid and formic acid increased and no accumulation of propionic acid was found in the presence of the AHL lactonase (Fig. 2b–d); (4) methanogenesis was not influenced by the AHL lactonase; (5) the bacterial community composition was altered by the AHL lactonase; (6) Gram-positive bacteria increased upon adding the AHL lactonase (Table 2 and Fig. 3); and (7) different bactericidal effects were found with or without the AHL lactonase; in particular, for Gram-negative and Gram-positive strains (Fig. 4 and Fig. S7). Among the results obtained, the differences in acidogenesis/acetogenesis, the increase in Gram-positive bacteria, and the bactericidal effect warrant further discussion.

The first point is that the methane fermentation was inhibited due to the low pH during the fermentation since organic acids (a kind of volatile fatty acid) can inhibit methanogenesis, and the inhibition is stronger at lower pH (Venkiteshwaran et al. 2015). Also, anaerobic digestion occurs optimally in the pH range of 6.8–7.2 and methane production decreases at less than pH 6.3 (Lay et al. 1998). In our study, the pH value during the fermentation was reduced by the effect of AHL lactonase (Fig. 2a). At the acidogenesis/acetogenesis stage, the concentration of three organic acids was increased by adding the AHL lactonase to WSS; acetic acid and formic acid (Fig. 2c, d) are important substrates for methane production (Ahring et al. 1995) and for hydrogen production (Barbosa et al. 2001; Kurokawa and Tanisho 2005). Reduced consumption of these two organic acids due to the low pH may be the main reason why acetic acid and formic acid accumulated. In addition, formic acid is an inhibitor of methane production (Ungerfeld 2015). Also, increasing acetic concentrations inhibits the degradation of propionic acid (Fukuzaki et al. 1990). Therefore, the small amount of propionic acid detected in WSS with the AHL lactonase could be due to the inhibition of its production. Based on the fact that AHL lactonase can catalyze cleavage of the AHL lactone ring (Grandclément et al. 2015), the difference in the production of organic acids may be due to the inactivation of AHL-based QS systems in Gram-negative bacteria.

For the second point, the bacterial community was changed by the addition of the AHL lactonase. Since the AHL lactonase itself does not have any bactericidal effect, it was expected that the bacterial community might be no different with or without the AHL lactonase (basically the AHL lactonase just inactivates the AHL-based QS system); therefore, the changes in the WSS community are an interesting discovery of this study. The percentage of two phyla, *Firmicutes* and *Bacteroidetes*, increased quickly in the presence of AiiM; these two phyla have been reported to have a role in digesting substrates in WSS (Regueiro et al. 2012). In addition, *Actinobacteria*, which increased in the presence of AiiM after 2 days, has cellulolytic activity (Lynd et al. 2002). We hypothesize that the inactivation of AHL-based QS systems for the Gram-negative bacteria through the AHL lactonase may change the production of antimicrobial molecules by the Gram-negative bacteria; thereby, the influence of the Gram-negative bacteria in the WSS should be reduced. In fact, microorganisms possess various mechanisms to produce bactericidal factors which inhibit or kill competitors. This competition naturally occurs among the microorganisms and plays an important role in ecological fitness. For the Gram-negative and Gram-positive bacteria, QS systems control the synthesis of these antimicrobial molecules (Dubuis et al. 2007; Duerkop et al. 2009). Namely, once AHL signals are degraded by the QQ enzyme, Gram-negative bacteria should not produce antimicrobial molecules; therefore, the influence of the Gram-negative bacteria in the microbial community is reduced. The influence of microbes that do not use AHL-based QS systems should increase if they are not sensitive to the antimicrobial molecules derived from the Gram-positive bacteria. We found that Gram-positive bacteria (in particular, *Firmicutes*) are dominant after the incubation of WSS with the AHL lactonase. Thus, the change in the microbial consortia causes the low pH during the fermentation since *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Proteobacteria* are acidogenic bacteria (Venkiteshwaran et al. 2015).

The last point is to understand is why the antimicrobial activities are different in the WSS with AHL lactonase. It was an interesting result that SPE-control targets only the Gram-positive bacteria whereas SPE-AiiM conversely inhibits Gram-negative bacteria (Fig. 4). The identification of antimicrobial molecules should be studied further because in the WSS, many types of antimicrobial molecules are produced. From the results of the bacterial community analysis, we found that two Gram-negative phyla, *Chloroflexi* and *Proteobacteria*, are reduced by the addition of AiiM to WSS. Therefore, the antimicrobial molecules produced by these two phyla in the absence of AiiM may inhibit the Gram-positive bacteria. As mentioned above, QS mechanisms induce the synthesis of antimicrobial secondary metabolites that are inhibitory to other bacteria, fungi, protozoa, and nematodes (Dubuis et al. 2007; Duerkop et al. 2009). For example, tetramic acid, which is produced by *P. aeruginosa*, a member of *Proteobacteria*, inhibits specifically Gram-positive bacteria (Kaufmann et al. 2005). In contrast, Gram-negative *Bacteroidetes* increased in the presence of AiiM, and this phylum can produce QQ enzymes (Mayer et al. 2015), and many genera from *Bacteroidetes* use AI-2 QS signaling instead of AHLs (Antunes et al. 2005; Peixoto et al. 2014). Hence, the antimicrobial activities from *Bacteroidetes* may inhibit Gram-negative bacteria utilizing AHL-based QS. In addition, antimicrobial activities from the two phyla, *Firmicutes* and *Actinobacteria*, may be essential to inactivate some of the Gram-negative bacteria. Bacteriocins derived from *Firmicutes* can inhibit Gram-

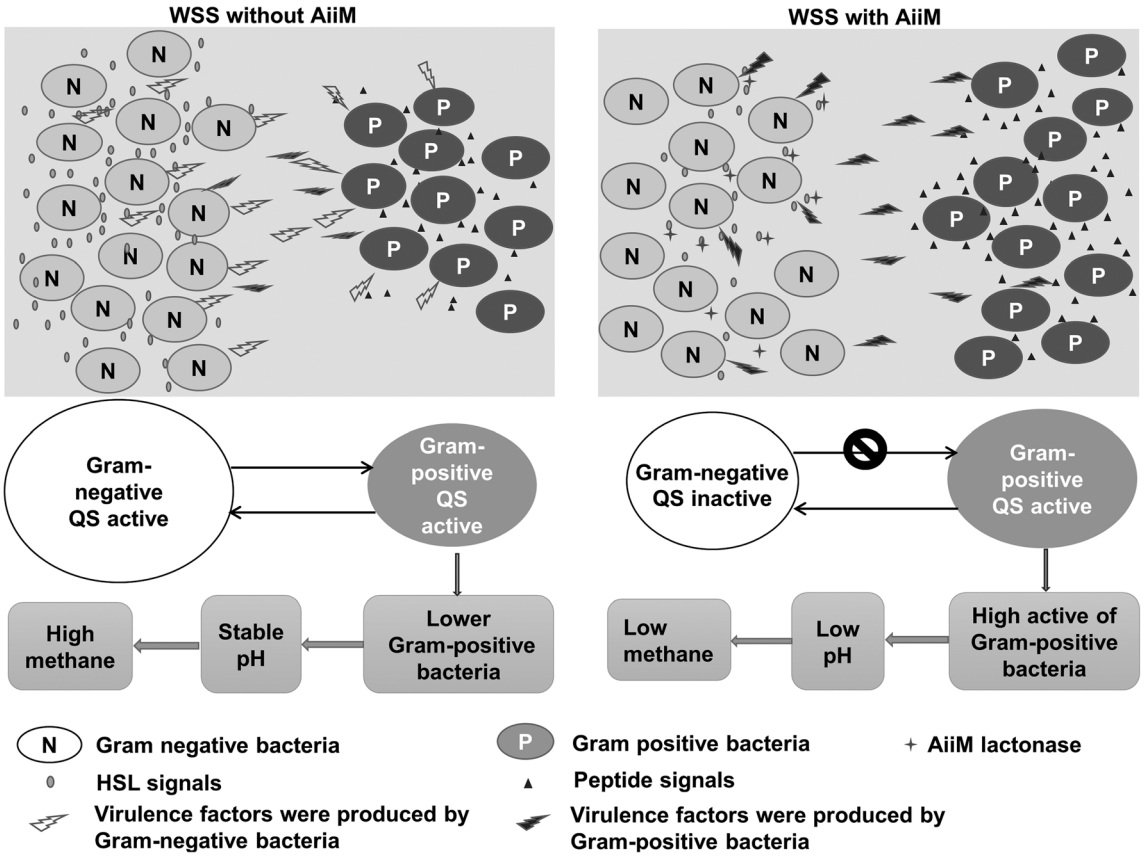

Fig. 5 The proposed model for the impact of AiiM QQ on the microbial community in the WSS anaerobic digestion process. In WSS without AiiM, Gram-negative bacteria and Gram-positive bacteria interact each other through QS and produce virulence factors to inhibit the growth of competitors. In this interaction, the percentage of Gram-positive bacteria is lower than that of Gram-negative bacteria. Consequently, the pH is stable and induces methane production. Upon the addition of AiiM lactonase, AiiM degrades the AHL signals generated by Gram-negative bacteria; therefore, the competitiveness of the Gram-negative bacteria decreases due to their reduced production of inhibitors of Gram-positive bacteria. Hence, the proportion of Gram-positive bacteria increases, the pH is decreased, and methane production is reduced

negative bacteria (Ananou et al. 2005; Smaoui et al. 2010) and *Actinobacteria* are a resource for antibiotics and QQ compounds against both Gram-negative bacteria and Gram-positive bacteria (Barka et al. 2016).

In summary, we propose that the mechanism for the impact of AHL lactonase on the anaerobic WSS fermentation is as shown in Fig. 5. The proposed mechanism is as follows: (1) the AHL lactonase inactivates AHL-based QS in some of the Gram-negative bacteria, (2) antimicrobial molecules from Gram-negative bacteria are reduced, (3) the bacterial community is changed as Gram-positive bacteria are favored, (4) the change in bacterial community reduces the pH during the fermentation, (5) methane-producing microbes are inactivated by the lower pH, and (6) methane production is reduced.

Supporting information

Methods for cloning AiiM lactonase, for assaying the quorum quenching activity of AiiM lactonase, and for SPE extraction and its quantitative assay are contained in the supplementary file. Also, the supplementary file also includes 7 supplementary figures and 1 table mentioned in main text of manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies performed with human participants or with animals by any of the authors.

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