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A layered structure of bacterial and archaeal communities and their in situ activities in anaerobic granules

Running title: A layered structure in anaerobic granules

By

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ABSTRACT

The microbial community structure and spatial distribution of microorganisms and their \textit{in situ} activities in anaerobic granules were investigated by 16S rRNA gene-based molecular techniques and microsensors for CH$_4$, H$_2$, pH, and oxidation-reduction potential (ORP). The 16S rRNA gene-cloning analysis revealed that the clones related to the phyla \textit{Alphaproteobacteria} (detection frequency of 51\%), \textit{Firmicutes} (20\%), \textit{Chloroflexi} (9\%), and \textit{Betaproteobacteria} (8\%) dominated the bacterial clone library and the predominant clones in the archaeal clone library were affiliated with \textit{Methanosaeta} (73\%). \textit{In situ} hybridization with the oligonucleotide probes at the phylum level revealed that these microorganisms were numerically abundant in the granule. A layered structure of microorganisms was found in the granule, where \textit{Chloroflexi} and \textit{Betaproteobacteria} were present in the outer shell of the granule, \textit{Firmicutes} was found in the middle layer, and aceticlastic \textit{Archaea} was restricted to the inner layer. Microsensor measurements for CH$_4$, H$_2$, pH, and ORP revealed that acid and H$_2$ production occurred in the upper part of the granule, below which H$_2$ consumption and CH$_4$ production were detected. Direct comparison of the \textit{in situ} activity distribution with the spatial distribution of the microorganisms implied that \textit{Chloroflexi} contributed to degradation of complex organic compounds in the outermost layer, H$_2$ was produced mainly by \textit{Firmicutes} in the middle layer, and \textit{Methanosaeta} produced CH$_4$ in the inner layer. We could also determine the effective diffusion coefficient for H$_2$ in the anaerobic granules to be $2.66 \times 10^{-5}$ cm$^2$ s$^{-1}$, which was 57\% in water.
INTRODUCTION

The upflow anaerobic sludge blanket (UASB) reactors are commonly used in the treatment of high-strength municipal and industrial wastewaters. Their design permits the retention of a greater amount of active biomass (known as granules) in comparison with other anaerobic reactors. These anaerobic granules harbor several metabolic groups of microorganisms involved in the anaerobic degradation of complex organic compounds, including hydrolytic, fermentative, syntrophic, and methanogenic microorganisms. These different trophic groups of anaerobes closely and coordinately interact each other within the granules and convert complex organic compounds in wastewaters into methane (CH\textsubscript{4}) and carbon dioxide (CO\textsubscript{2}). The microbial communities of anaerobic granules treating different wastewaters have been investigated by culture-independent 16S rRNA gene-based molecular analyses (14, 22, 36), which allowed one to obtain more complete inventories of microorganisms in anaerobic granules. The results have shown that anaerobic granules consisted of a phylogenetically diverse group of microorganisms; however, the majority of them have not yet been cultivated (14, 22, 36).

The application of fluorescence in situ hybridization (FISH) with specific oligonucleotide probes further allows us to determine the abundance and in situ spatial distribution of specific phylogenetic groups in anaerobic granules (14, 20, 35, 41). By using the FISH technique, distinct multi-layered structures of different phylogenetic groups of microorganisms have been determined in different anaerobic granules cultivated on different substrates (20, 22, 35). These well-organized unique structures are thought to be a result of sequential degradation of complex organic compounds by each different trophic group within the granules, although it has not yet been confirmed experimentally.
In addition, spatial distribution of the microorganisms obtained from FISH analysis does not necessarily reflect microbial activity distributions. This is because the majority of microorganisms have not yet been cultivated, and phylogeny and phenotypes are not necessarily congruent with physiology. The spatial distributions of \textit{in situ} metabolic functions in anaerobic granules have been poorly understood mainly due to a lack of specific analytical tools with a sufficient spatial resolution. Only a few studies have analyzed the distributions of glucose-degrading, fermentative, and sulfate-reducing activities in UASB granules by using microsensors for glucose, pH, and hydrogen sulfide (H$_2$S) (13, 21, 42). In these studies, CH$_4$ production rates were, however, not directly measured with microsensors. Santegoeds et al. (34) used CH$_4$ biosensors and H$_2$S microsensors to directly measure \textit{in situ} methanogenic and sulfate-reducing activities in anaerobic granules, and then related them to the spatial distributions of methanogenic and sulfate-reducing bacterial populations. They demonstrated a distinct layered structure of microbial activities, in which sulfate reduction occurred in the outer layer, whereas CH$_4$ production was found in the center of the granules. However, they did not investigate the \textit{in situ} distributions of fermentative and syntrophic populations and their activities in the granules, and hence the overall conversion mechanism of complex organic compounds to CH$_4$ within granules has not been elucidated.

To investigate the overall anaerobic conversion mechanism of organic compounds to CH$_4$ within anaerobic granules, we first analyzed the bacterial and archaeal community structures by 16S rRNA gene-cloning analysis, and then the spatial distribution of important phylogenetic groups in the granules was determined by fluorescence \textit{in situ} hybridization (FISH). Second, we applied the microsensors for CH$_4$, H$_2$, pH, and oxidation-reduction
potential (ORP) to determine the spatial distribution of the in situ fermentative (H⁺-producing), syntrophic (H₂-producing), and methanogenic activities in the granules. The microbial activity distribution was directly compared with the spatial distribution of the microorganisms.

6 MATERIALS AND METHODS

7 Sludge source.

Anaerobic granular sludge was collected from the bottom of a lab-scale upflow anaerobic sludge blanket (UASB) reactor (height, 50 cm; diameter, 5 cm) operated at 35°C. The reactor was inoculated with 0.7 liter of anaerobic granular sludge obtained from a full-scale UASB reactor treating the wastewater from an isomerized sugar-processing plant. The lab-scale reactor was fed with a synthetic medium at an average organic loading rate of 1.67 g chemical oxygen demand (COD) l⁻¹ day⁻¹ and a hydraulic residence time (HRT) of 8.2 h. The synthetic medium contained skim powdered milk (1,250 mg l⁻¹) as carbon and energy sources, NaHCO₃ (1,000 mg l⁻¹), K₂HPO₄ (50 mg l⁻¹), and the mineral solution (4). Granule samples were obtained from the lab-scale UASB reactor after 1 year of operation. The water qualities of the effluent during the sampling period were as follows (average ± standard deviation, n = 31): COD, 30 ± 9 mg l⁻¹; COD removal rate, 95 ± 1%; ORP, -230 ± 20 mV; gas production rate, 0.10 ± 0.02 liter h⁻¹; CH₄ content in gas, 55 ± 15%; CO₂ content in gas, 11 ± 4%; N₂ content in gas, 34 ± 17%; H₂ content in gas, 2300 ± 3300 ppm; acetate, 165 ± 81 µM; propionate, 77 ± 46 µM; and butyrate, 5.5 ± 7.7 µM. Other volatile fatty acids (butyrate, isobutyrate, and formate) were sometimes detected at trace levels.
DNA extraction and PCR amplification.

The granule samples were homogenized in the sterilized, distilled water and approximately 1.0-ml subsamples were subjected to DNA extraction. Total DNA was extracted from the homogenized granules with a FastDNA spin kit for soil (Bio 101; Qbiogene, Inc., Carlsbad, CA) as described in the manufacturer’s instructions. 16S rRNA gene fragments were amplified from the extracted total DNA with Taq DNA polymerase (TaKaRa Bio, Inc., Ohtsu, Japan) by using a primer set of 11f (19) and 1492r (39) for bacterial community, and a primer set of A109f (40) and 1492r (39) for archaeal community. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel and purified with a WIZARD PCR Preps DNA purification system (Promega). To reduce the possible PCR bias, the 16S rRNA gene was amplified in 6 to 10 PCR tubes and all tubes were combined for the next cloning step.

Cloning and sequencing of the 16S rRNA genes and phylogenetic analysis.

The purified PCR products were ligated into a qCR-XL-TOPO vector with a TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA). The ligated products were transformed into TOP10-competent Escherichia coli cells (Invitrogen). Plasmids were extracted from the cloned cells and purified with a Wizard Plus Minipreps DNA purification system (Promega). Nucleotide sequencing was performed with an automatic sequencer (3100 Avant genetic analyzer; Applied Biosystems). All sequences were checked for chimeric artifacts by using a CHECK_CHIMERA program from the Ribosomal Database Project (24). Partial sequences (approximately 500 bp) were compared with similar sequences of the reference organisms by a BLAST search (1). Sequences with 97% or greater similarity were grouped
into operational taxonomic units (OTUs) by a SIMILARITY MATRIX program from the Ribosomal Database Project (24). Nearly complete sequencing of the 16S rRNA gene of each representative OTU was performed, and the sequences were aligned with a CLUSTAL W package (37). The Molecular Evolutionary Genetics Analysis (MEGA) program, version 3.1, was used to construct phylogenetic trees based on the neighbor-joining (33) and maximum-parsimony methods. Bootstrap resampling analysis of 1,000 replicates was performed to estimate the degree of confidence in tree topologies.

Fixation and cryosectioning of granule samples.

Granule samples were fixed in 4% paraformaldehyde solution for 8 h at 4°C, washed three times with phosphate-buffered saline (10 mM sodium phosphate buffer, 130 mM sodium chloride [pH 7.2]), and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) overnight to infiltrate the OCT compound into the granule, as described previously (29). After rapid freezing at -21°C, 20-µm-thick slices were prepared with a cryostat (Reichert-Jung Cryocut 1800, Leica, Bensheim, Germany).

Fluorescent in situ hybridization.

In situ hybridization was performed according to the procedure described by Amann (2) and Okabe et al. (29). The following 16S and 23S rRNA-targeted oligonucleotide probes were used: EUB338 (3), EUB338 II (8), EUB338 III (8), ARC915 (31), ALF968 (28), BET42a (25), GNSB-941 (16), HGC69A (32), LGC354A (27), LGC354B (27), LGC354C (27), MB1174 (31), MG1200 (31), MS821 (31), and MX825 (31). The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC).
The phylum-specific probes were applied simultaneously with the ARC915 probe specific for *Archaea*. A model LSM510 confocal laser-scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (488 nm) and HeNe laser (543 nm) was used.

Microsensor measurements.

For microsensor measurements, the granules with ca. 2 mm in diameter were selected and positioned using five insect needles in the flow cell reactor (4.0 liter) that was filled with the synthetic medium used for the lab-scale UASB reactor at 35°C. The medium in the flow cell reactor was kept anaerobic by adding a reducing agent (thioglycolic acid at 100 mg l⁻¹) and by continuous bubbling with N₂, which also resulted in sufficient mixing of the medium. The ORP of the medium was ca. -200 mV. An average liquid velocity, judged from movement of suspended particles, was ca. 5 mm s⁻¹. The granules were acclimated in the medium for at least 6 h before measurement to ensure that steady-state profiles were obtained. The *in situ* steady-state concentration profiles of CH₄, H₂, pH, and ORP in the granules were measured using microsensors as described by Okabe et al. (30). At least three concentration profiles were measured for each chemical species. For practical reasons, a concentration profile was measured only once in a granule, therefore, concentration profiles of different chemical species were measured in different granules.

Microscale biosensors for CH₄ were constructed as described by Damgaard and Revsbech (11). Because all measurements were performed under anoxic conditions, an oxygen-scavenging guard capillary (12) was not applied. Hence, the CH₄ microsensor was assembled from an oxygen microsensor, a gas capillary, and a medium capillary. The tip
diameters of the microsensors were 50-100 µm. A culture of the methane-oxidizing bacterium, Methylosinus trichosporium (ATCC 49243), was injected into the medium capillary. The principle of the CH₄ microsensor is based on a counter diffusion of oxygen and CH₄. CH₄ diffusing through the membrane of the medium capillary is consumed by the methane-oxidizing bacteria with a concomitant decrease of oxygen inside the reaction space (i.e., the medium capillary). Oxygen consumed is detected by the internal oxygen microsensor and translated into a CH₄ partial pressure by calibration. Calibration was routinely performed before and after a measurement by placing a CH₄ microsensor in a calibration chamber (100 ml) into which CH₄ and N₂ gases were continuously blown at known flow rates at 35°C. After the sensor signal stabilized, the signal was monitored and the CH₄ concentration was measured by a gas chromatography. CH₄ concentration was changed stepwise by changing the flow rates of CH₄ and N₂ gases. This procedure was repeated over the full range of 0 to 100% CH₄ saturation. The response to 0% CH₄ saturation (i.e., 100% N₂ saturation) was typically between 70 and 90 pA, and 90% response times were ca. 200 s. The response was linear ($r^2 > 0.95$) over the range of 0 to 100% CH₄ saturation. The slope of the calibration curves was ca. 20 pA per atm of CH₄ (see Fig. S1 in the supplemental material). Sensor life span was ca. 2 weeks. If the slope of the calibration curve was less than 10 pA per atm of CH₄, the microsensor was discarded.
Fig. S1. A typical calibration curve for a microscale biosensor for methane. The solid line indicates linear regression. The equation of the straight line was $y = -19x + 90$ with $r^2 = 0.98$.

Polarographic H₂ microsensors were constructed as described by Ebert and Brune (15). The tip diameters of the microsensors were ca. 10 µm, 90% response times were less than 2 s, and the detection limit was ca. 1 µM. Calibration was routinely performed by immersing a microsensor in a calibration chamber filled with the synthetic medium which continuously bubbled with H₂ and N₂ gases. The H₂ concentration in the medium was measured by a gas chromatography. H₂ concentration was changed stepwise by changing the flow rates of H₂ and N₂ gases. ORP microsensors, which were made from a platinum wire coated with a glass micropipette, were constructed and calibrated as described by Jang et al. (18). All ORP data reported in this paper were the potential difference measured between an ORP microsensor
and the Ag/AgCl reference electrode. Potentiometric pH microsensors were constructed, calibrated, and used according to the protocol as described by Okabe et al. (29).

Microbial activity calculations.

Net volumetric CH$_4$ and H$_2$ production rates (R(CH$_4$) and R(H$_2$), respectively) in the granule were estimated from the steady-state concentration profiles of CH$_4$ and H$_2$ by using Fick’s second law of diffusion as previously described by Santegoeds et al. (34) and Lorenzen et al. (23). Furthermore, the total H$_2$ production rate from the granule was calculated using Fick's first law of diffusion (29). Molecular diffusion coefficients ($D_{w}$) of $1.49 \times 10^{-5}$ cm$^2$ s$^{-1}$ for CH$_4$ and $4.50 \times 10^{-5}$ cm$^2$ s$^{-1}$ for H$_2$ in water at 25°C were used for the calculations (7). Molecular diffusion coefficients in water at 35°C ($D_{w35}$) were calculated according to the Stokes-Einstein relationship (7). Furthermore, effective diffusion coefficient ($D_{eff}$) of these compounds in the granules were estimated by correcting $D_{w}$ with the ratio of the diffusivities in granules and in water (57%), as determined with the H$_2$ microsensor in this study (see Discussion).

Estimation of effective diffusion coefficient ($D_{eff}$).

Granules with a diameter of ca. 2 mm were selected and deactivated in pure chloroform for 10 min. After being rinsed, the deactivated granule was mounted in the flow cell reactor containing the synthetic medium for microsensor measurements at 35°C, and a H$_2$ microsensor was positioned in the center of the granule with a micromanipulator. After preincubation for at least 30 min, H$_2$ gas was bubbled. The evolution of the H$_2$ concentration in the center of the granule was monitored over time in triplicate, and the average $D_{eff}$ was
calculated according to the protocol as described by Cronenberg and van den Heuvel (6).

Chemical analyses.

The concentrations of chemical oxygen demand (COD) were determined according to the standard methods (5). Volatile fatty acids were determined by high-performance liquid chromatography (LC-10AD system; Shimadzu Co., Kyoto, Japan) equipped with a Shimadzu Shim-pack SCR-102H column (0.8 by 30 cm) after filtration with 0.2-µm-pore size membranes (Advantec Co., Ltd., Tokyo, Japan). CH$_4$, H$_2$, and CO$_2$ in gaseous samples were determined by gas chromatography (GC-14B; Shimadzu Co., Kyoto, Japan) equipped with a thermal-conductivity detector and a Shincarbon-ST column (Shimadzu Co., Kyoto, Japan). ORP and pH were directly determined using an ORP and a pH electrode, respectively.

Nucleotide sequence accession numbers.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the clones used for the phylogenetic analysis are AB329636 to AB329664.
Results

Bacterial and archaeal community structures.

16S rRNA gene clone libraries of Bacteria and Archaea were constructed from the anaerobic granule samples taken from the UASB reactor. From the Bacteria clone library 105 clones were randomly selected and the partial sequences of about 500 bp were analyzed. In total, the clones were grouped into 25 OTUs on the basis of more than 97% sequence similarity within an OTU. Then, a nearly complete 16S rRNA gene sequence of a representative clone of each OTU was analyzed. The phylogenetic trees were constructed by neighbor-joining and maximum-parsimony methods, and both methods resulted in essentially the same topology. The phylogenetic tree inferred by the neighbor-joining method is shown in Fig. 1. The distribution of cloned sequences among phyla is as follows: Alphaproteobacteria, 51%; Firmicutes, 20%; Chloroflexi, 9%; Betaproteobacteria, 8%; Actinobacteria, 4%; Deltaproteobacteria, 3%; Cyanobacteria, 2%; Gammaproteobacteria, 2%; Epsilonproteobacteria, 1%; and Bacteroidetes, 1%. The most frequently detected clones (40 of 105 clones) were represented by OTU 8, which was closely related to the Sphingomonas rhizogenes (99.8% sequence similarity).
Fig. 1. Phylogenetic tree representing the affiliation of the 16S rRNA clone sequences of *Bacteria* retrieved from granule samples (OTU#). The tree was generated by using nearly full length of 16S rRNA gene sequences and the neighbor-joining method. The scale bar represents 5% estimated divergence. The numbers at the nodes are bootstrap values (1,000 replicates) with more than 50% bootstrap support.
For the Archaea clone library, 48 clones were randomly selected and partial sequences of about 500 bp were analyzed. The clones were grouped into 4 OTUs. The clones belonging to OTUs 1 and 4 were related to Methanosaeta whereas the clones belonging to the other OTUs (OTUs 2 and 3) were related to Methanomicrobiales. Their representative sequences were used for phylogenetic analysis (Fig. 2). The most frequently detected clones (33 of 48, detection frequency of 69%) represented by OTU 1 were affiliated with Methanosaeta concilii with 98.3% sequence similarity. The clones represented by OTU 4 (2 of 48, detection frequency of 4%) were affiliated with Methanosaeta harundinacea with 99.6% sequence similarity. The clones represented by OTU 2 (9 of 48, detection frequency of 19%) and OTU 3 (4 of 48, detection frequency of 8%) were related to the orders Methanomicrobiales with 96.2% and 97.8% sequence similarities, respectively. These results indicated that diversity of the Archaea clone library was lower than for the Bacteria.
Fig. 2. Phylogenetic tree representing the affiliation of the 16S rRNA clone sequences of *Archaea* retrieved from granule samples (OTU#). The tree was generated by using nearly full length of 16S rRNA gene sequences and the neighbor-joining method. The scale bar represents 5% estimated divergence. The numbers at the nodes are bootstrap values (1,000 replicates) with more than 50% bootstrap support.

Spatial distribution of microorganisms in granules.

Cross-sectional differential interference contrast (DIC) images of the granule showed that the granule had a multi-layered structure consisting of biomass and interstitial voids (Fig. 3A). Analysis of the granule after staining with DAPI showed microorganisms were predominantly present in the outer 400 µm (Fig. 3A). It is most likely that the dark
nonstaining center consisted of inert matter and dormant microbial cells. This is probably attributed to substrate limitation in the center of the granules due to the relatively low COD loading rate in the reactor analyzed. The nonstaining center was always observed in the granules analyzed with diameters exceeding about 1,000 µm. FISH using FITC-labeled EUB338-mixed probe and TRITC-labeled ARC915 probe revealed that the outer layer (ca. 250-µm-thick) was dominated by bacterial cells whereas the inner layer (below 250 µm from the surface) was occupied mainly by archaeal cells (Fig. 3B). Archaeal and bacterial signals were low in the granule interior (below ca. 400 µm from the surface). This layered structure was repeatedly observed in all of the granular sections analyzed. Filamentous cells were observed in the uppermost layer of the granules and these cells were hybridized with the probe GNSB-941 specific for almost all members of the phylum Chloroflexi (Fig. 3C). The BET42a-stained cells were also present in the outer shell of the granule (Fig. 3D). The cells hybridized with the probe LGC354 specific for Firmicutes were numerically most abundant Bacteria in the inner layer of the granule (Fig. 3E). The abundance and fluorescent intensity of ALF968-stained cells were low (Fig. 3F) although the bacterial clone library was predominated by the members of Alphaproteobacteria (Fig. 1). The MX825-stained cells surrounded the dense spherical microcolonies that composed of a number of coccoid cells stained with the probe HGC69A (i.e., Actinobacteria) in the middle layer (at a depth of ca. 200 µm) of the granules (Fig. 3G). Among archaeal cells, the MX825-stained cells (i.e., Methanosaeta) predominated while the remaining portion of archaeal cells could not be hybridized with the MG1200, MB1174, and MS821 probes (data not shown).
Fig. 3 Confocal laser scanning microscope images of thin sections of the anaerobic granules showing the *in situ* spatial organization of bacteria and archaea. (A) Staining with DAPI and differential interference contrast (DIC) image. (B) FISH with TRITC-labeled probe
ARC915 (red) and FITC-labeled EUB338-mixed probe (green). (C) FISH with FITC-labeled probe GNSB-941. (D) FISH with TRITC-labeled probe ARC915 (red) and FITC-labeled probe BET42a (green). (E) FISH with TRITC-labeled probe ARC915 (red) and FITC-labeled probe LGC354 (green). (F) FISH with TRITC-labeled probe ARC915 (red) and FITC-labeled probe ALF968 (green). (G) FISH with TRITC-labeled probe MX825 (red) and FITC-labeled probe HGC69A (green). Scale bars indicate 200 μm (A, B) and 50 μm (C-G).

Concentration profiles and spatial distribution of microbial activities.

Steady-state concentration profiles of ORP, pH, H₂, and CH₄ in the granules are shown in Fig. 4. pH decrease (i.e., acid production) was found in the upper part of the granule and below which pH increased (Fig. 4A). When H₂ profiles were measured in the medium without NaHCO₃, H₂ profile showed a peak of 26 μM at a depth of 100 μm and H₂ concentration readily decreased in the inner layer of the granule (Fig. 4B). The addition of 12 mM of NaHCO₃ stimulated H₂ consumption activity and H₂ concentration in the granule became under detection limit (1 μM) (data not shown). CH₄ concentration gradually increased throughout the granule and its gradient was steeper in the outer layer of the granule (Fig. 4B).
**Fig. 4.** Concentration profiles of pH, ORP, CH₄ and H₂ in the anaerobic granule. The profiles are average values (n = 3) and error bars represent the standard deviations of triplicate measurements. Zero on the vertical axis corresponds to the surface of the granule.

The spatial distributions of R(CH₄) and R(H₂) were calculated on the basis of the measured profiles. **Fig. 5** shows that anaerobic processes occurred in distinctly different layers within the granule. H₂ production was exclusively detected at a depth of 100 µm. H₂ produced was partly consumed below 300 µm from the surface and emitted from the granule with the total production rate of 2.3 ± 4.3 µmol cm⁻² h⁻¹. CH₄ was produced mainly in the inner layer (below ca. 300 µm from the surface) with the maximum rate of 11.5 ± 3.9 µmol.
cm$^{-3}$ h$^{-1}$ at a depth of 300 µm.

Fig. 5. Spatial distribution and magnitude of the net volumetric production rates of CH$_4$ and H$_2$. The rates were calculated based on the corresponding concentration profiles shown in Fig. 4. The profiles are average values ($n = 3$) and error bars represent the standard deviations of triplicate measurements. Negative values indicate consumption rates. Zero on the vertical axis corresponds to the surface of the granule.

Transient measurements for determination of effective diffusion coefficient ($D_{eff}$)

For the determination of $D_{eff}$, continuous monitoring of H$_2$ concentrations at the center of the granule deactivated by pure chloroform was performed. The ratio of H$_2$ concentration at the granule center to one in the bulk liquid ($C/C_b$) was calculated, and a representative profile of $C/C_b$ transient is shown in Fig. 6. A mathematical model that describes the $C/C_b$ transient in the granule provided a good fit to the profile measured (correlation coefficient
of 0.98). Based on the model, $D_{eff}$ for H$_2$ in the granules were determined to be $2.66 \pm 0.13 \times 10^{-5}$ cm$^2$ s$^{-1}$ (average ± standard deviation, n = 3).

**Fig. 6.** A typical transient H$_2$ concentration profile measured at the center of the granule inhibited by chloroform. Points indicate H$_2$ concentrations measured and a solid line is a theoretical curve.

**Discussion**

The microsensor measurements indicated a distinct layered structure of the microbial activities in the anaerobic granule, with net acid (H$^+$) and H$_2$ production (i.e., fermentative and syntrophic activities) at a depth of 100 µm, and net H$_2$ consumption and CH$_4$ production (i.e., methanogenesis) below 300 µm from the surface (**Fig. 5**). Because anaerobic degradation of organic compounds is a multi-step process, a layered structure of the bacteria that hydrolyze complex organic compounds in wastewater to fundamental structural building blocks (e.g., glucose and amino acids) at the granule surface, fermentative bacteria that ferment these products to fatty acids and subsequently syntrophic bacteria oxidizing
fatty acids and alcohols to H$_2$ and acetate in the middle layer, and methane-producing archaea in the inner layer, was developed. Our knowledge of the spatial distribution of microbial activities, especially CH$_4$-producing activity in anaerobic granules is very limited because *in situ* activity measurements require specific analytical tools (e.g., microsensors). CH$_4$ microprofiles and the distributions of CH$_4$ production activity in a sewage biofilm (10), a rice paddy soil (12), and a lake sediment (9) have been measured with micrometer resolution by using CH$_4$ microsensors. Only one study indicated CH$_4$ microprofiles in anaerobic granules (34). This study demonstrated a similar layered structure of the microbial activities in methanogenic-sulfidogenic aggregates in which sulfidogenic activity was found in the outer layer and CH$_4$ production only started from 300 µm onwards inside the aggregate. In our study, although a sulfate reduction zone was not analyzed, sulfate reduction rate could be very low because sulfate concentration was less than 2 µM in the synthetic medium for cultivation and microsensor measurements.

*In situ* hybridization results showed that the outer layer (ca. 250-µm-thick) of the granule was dominated by *Bacteria* whereas the inner layer (below 250 µm from the surface) consisted of *Archaea* (Fig. 3B). Similar layered structures of microorganisms in anaerobic granules have been reported elsewhere (20, 35). Direct comparison of the microsensor results with the FISH ones revealed that H$_2$ might be produced at a depth of 100 µm mainly by members of *Firmicutes*, and *Archaea* affiliated with *Methanosaeta* produced CH$_4$ below 300 µm. Phylogenetic analysis revealed that members of the phyla *Alphaproteobacteria*, *Firmicutes*, *Chloroflexi*, and *Betaproteobacteria* dominated the bacterial clone library. This result was in good agreement with the FISH ones. *Chloroflexi* was mainly detected in the granule surface (Fig. 3C), indicating that they contributed to the
hydrolysis of complex organic compounds. Ariesyady et al. (4) analyzed in situ function (i.e., glucose-, propionate-, butyrate-, and acetate-degrading activities) of bacteria in a full-scale anaerobic sludge digester by microautoradiography (MAR)-FISH technique, and revealed that *Chloroflexi* was one of the numerically dominant glucose-degrading bacterial groups. The filamentous *Chloroflexi*-like bacteria also contributed to coating of other microorganisms and formation of dense and compact granules. The bacteria belonging to *Firmicutes* have been detected in methanogenic granules (14, 22). These bacteria can anaerobically utilize glucose, propionate, butyrate, and acetate (4), and produce H\(_2\) (17). In contrast, Ariesyady et al. (4) demonstrated that glucose-utilizing rate of *Betaproteobacteria* was very low and *Alphaproteobacteria* did not utilize all of the substrates tested (i.e., glucose, propionate, butyrate, and acetate) in an anaerobic sludge digester, indicating less contribution of these bacteria to the degradation of organic compounds in the granules.

The clones affiliated with aceticlastic *Methanosaeta* were frequently detected in the archaeal clone library (Fig. 2), and the cells hybridized with the probe MX825 specific for the genus *Methanosaeta* were the dominant member of *Archaea* in the granule. In general, aceticlastic methanogens are more abundant than hydrogenotrophic ones in methanogenic consortia (14, 35). Although the hydrogenotrophic methanogens affiliated with *Methanomicrobiales* were identified by phylogenetic analysis, the number of hydrogenotrophic methanogens hybridized with probes MG1200 and MB1174 was under detection limit in this study. The probe MG1200 is specific for *Methanomicrobiales* whereas the probe MB1174 is specific for *Methanobacteriales*. Moreover, the 16S rRNA gene sequence of the clones belonging to OTU2 in the *Archaea* clone library, which were more frequently detected than those belonging to OTU3, had two mismatches with the probe
MG1200 sequence. Therefore, further studies are urgently needed to identify the hydrogenotrophic methanogens with additional oligonucleotide probes for hydrogenotrophic methanogens, and evaluate the significance of them in methane production.

We could determine the effective diffusion coefficient ($D_{\text{eff}}$) for H$_2$ in the anaerobic granules with H$_2$ microsensors. In this study, $D_{\text{eff}}$ for H$_2$ in the granules at 35°C was $2.66 \pm 0.13 \times 10^{-5}$ cm$^2$ s$^{-1}$, which was 57 ± 3% of the molecular diffusion coefficient for H$_2$ in water ($D_{w,35}$; $4.65 \times 10^{-5}$ cm$^2$ s$^{-1}$). The similar ratios were reported for glucose by applying microsensors to anaerobic granules (21, 34). Based on the results, the $D_{\text{eff}}$ for CH$_4$ and H$_2$ were assumed to be 57% of their $D$ in water, and these values were used to calculate the microbial activities (R) in the granules (Fig. 5). The calculated CH$_4$ production rates in the granule (11.5 µmol cm$^{-3}$ h$^{-1}$) were comparable to those in the methanogenic aggregates (ca. 15 µmol cm$^{-3}$ h$^{-1}$) (34) and were two orders of magnitude higher than those in a sewage biofilm (ca. 0.18 µmol cm$^{-3}$ h$^{-1}$) (10). High CH$_4$ production activity of the anaerobic granules might be attributed to higher abundance of methanogens.

Maximum H$_2$ concentration (26 µM) in the granule (Fig. 4B) was one order of magnitude higher than that in anaerobic aggregates (less than 5 µM) (34). In contrast, these values were lower than those in the hindgut of a wood-feeding lower termite (ca. 50 µM) (15). In methanogenic consortia, H$_2$ produced by syntrophic bacteria should be efficiently consumed because the oxidation of intermediary metabolites (e.g., fatty acids and alcohols) into H$_2$ is thermodynamically feasible only at very low H$_2$ partial pressures (e.g., between $10^{-4}$ and $10^{-6}$ atm for anaerobic propionate degradation) (26). To investigate whether H$_2$ concentrations in the granule analyzed in this study were low enough to carry out the
syntrophic H$_2$-producing reaction (e.g., propionate oxidation), the relationship between H$_2$
concentration and the Gibbs free energy change ($\Delta G$) for propionate oxidation was
calculated according to the following equation (38).

$$\Delta G = \Delta G_0' + RT \ln \left( [H_2]^3 \frac{[Ace]}{[Pro]} \right)$$

where $\Delta G$ is the actual free energy, $\Delta G_0'$ is the standard free energy (76.5 kJ mol$^{-1}$), R is the
ideal gas constant, T is the temperature (K), and [H$_2$], [Ace], and [Pro] are the molar H$_2$,
acetate, and propionate concentrations, respectively. The average concentrations of
propionate (77 µM) and acetate (165 µM), and temperature (35°C) in the lab-scale UASB
reactor analyzed were used for the calculation. The H$_2$ concentration for $\Delta G = 0$ was
calculated to be 36.8 µM, indicating that the H$_2$ concentration in the granule (< 26 µM) was
low enough for the syntrophic degradation to proceed.

There was a difficulty with microsensor measurements. It was curious that low but
significant CH$_4$-producing activities were detected in the center of the granule in which the
density of Archaea was low. A possible explanation could be compression of the granule by
inserting the CH$_4$ microsensor, because its tip diameter was relatively large (50-100 µm)
and conical as compared with those of other microsensors. Therefore, CH$_4$ concentration
profiles were monitored by advancing the microsensor in steps of 300 µm (Fig. 4B).

In conclusion, combining the 16S rRNA gene-based molecular techniques with
microsensors provided direct information about phylogenetic diversities, spatial
distributions, and activities of Bacteria and Archaea in anaerobic granules. The spatial
distributions of the microorganisms and their in situ activities in the granule were
characterized by a distinct layered structure. Acid and H$_2$ production occurred in the upper
part of the granule, below which H$_2$ consumption and CH$_4$ production were found.
Chloroflexi might contribute to the hydrolysis of complex organic compounds in the outer shell of the granule, H₂ was produced mainly by members of Firmicutes in the middle layer, and Archaea affiliated with Methanosaeta produced CH₄ in the inner layer. Further studies with the microsensor for volatile fatty acids and additional oligonucleotide probes for syntrophic bacteria and hydrogenotrophic methanogens are needed for better understanding of the overall degradation mechanism of complex organic matter in anaerobic granules.

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