Characterization of microbial community structures and their activities in single anaerobic granules by beta imaging, microsensors and fluorescence *in situ* hybridization

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ABSTRACT

The spatial distribution of microorganisms and their *in situ* activities in anaerobic granules were investigated by fluorescence *in situ* hybridization (FISH), beta imaging and microsensors. FISH results revealed a layered structure of microorganisms in the granule, where *Chloroflexi* was present in the outermost layer, *Smithella* spp. and *Syntrophobacter* spp. were found in a depth of ca. 100 μm, and *Archaea* was restricted to the inner layer (below ca. 300 μm from the surface). Substrate uptake patterns elucidated by beta imaging demonstrated that glucose uptake was highest at 50 μm depth, whereas propionate uptake had a peak at 200 μm depth. In addition, microsensor measurements revealed that acid was produced mainly at 100 μm depth and H_2 production was detected at a depth from 100 to 200 μm. H_2 consumption and corresponding CH_4 production were found below 200 μm from the surface. Direct comparison of these results implied sequential degradation of complex organic compounds in anaerobic granules; *Chloroflexi* contributed to fermentation of organic compounds and acid production in the outermost layer, volatile fatty acids were oxidized and H_2 was produced mainly by *Smithella* spp. and *Syntrophobacter* spp. at a depth from 100 to 200 μm, and *Archaea* produced CH_4 below ca. 300 μm from the surface.

Key words | beta imaging, FISH, microbial activity, microbial community structure, microsensors

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INTRODUCTION

The upflow anaerobic sludge blanket (UASB) reactors are one of the most common types of bioreactors used in the treatment of high-strength municipal and industrial wastewaters. They rely primarily on the degradation of organic matter by granular sludge under anaerobic conditions. These anaerobic granules harbor several metabolic groups of microorganisms involved in the anaerobic degradation of complex organic compounds, including hydrolytic, fermentative, acidogenic, syntrophic, and methanogenic microorganisms, which enables the organic compounds to be degraded step-by-step into methane (CH_4) and carbon dioxide (CO_2).

Diversity of microbial communities in anaerobic granules treating different wastewaters has been investigated by molecular analyses based on the 16S rRNA gene, which allowed one to obtain more complete inventories of microorganisms in anaerobic granules. For example, Díaz *et al.* (2006) analyzed microbial compositions of methanogenic granules from an anaerobic bioreactor treating wastewater of a beer brewery by molecular microbiological techniques and demonstrated that *Firmicutes*, *Nitrospira* and *Deferribacteres* were the predominant *Bacteria* in the granules. Furthermore, fluorescence *in situ* hybridization (FISH) with the oligonucleotide probes demonstrated *in situ* spatial distribution and abundance of microorganisms in anaerobic granules (Sekiguchi *et al.* 1999; Lanthier *et al.* 2002; Díaz *et al.* 2006). By using the FISH technique, a distinct layered structure of microorganisms has been determined in different anaerobic granules cultivated on different substrates (Santegoeds *et al.* 1999; Sekiguchi *et al.* 1999; Lanthier *et al.* 2002; Satoh *et al.* 2007). These

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investigations considerably improved our understanding of the microbial community structure in anaerobic granules. 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.

Sequential degradation of complex organic compounds in anaerobic granules has been poorly understood as compared with *in situ* spatial distribution of microorganisms mainly due to a lack of specific analytical tools with a sufficient spatial resolution. So far, only a few studies have analyzed glucose-degrading, sulfate-reducing, and methanogenic activities in UASB granules by using microsensors for glucose, hydrogen sulfide (H_2S), and methane (CH_4) (Lens *et al*. 1993; Santegoeds *et al*. 1999). Previously, we also used the microsensors for CH_4, hydrogen (H_2) and pH and investigated *in situ* hydrogen-producing and consuming, and methane-producing activities in anaerobic granules (Satoh *et al*. 2007). However, in the previous studies, the sequential degradation processes occurring in anaerobic granules were partly determined in each study and there is no study to comprehensively elucidate the sequential degradation processes from fermentation of complex organic compounds to methane production.

In this study, the comprehensive pathway of the anaerobic conversion (from degradation of complex organic compounds to CH_4 production) within single anaerobic granules was investigated. The spatial distribution of the *in situ* acid-producing, hydrogen-producing and consuming, and methane-producing activities in the granules were determined with the microsensors for CH_4, H_2, pH and oxidation-reduction potential (ORP). Because construction of microsensors for glucose and propionate is difficult, fermentative (i.e. glucose-degrading) and syntrophic (i.e. propionate-consuming) activities in the granules were determined by beta imaging. The beta imaging has been used in medical fields as a sensitive technique to measure the two-dimensional distribution of incorporated radiotracer in tissue samples with high spatial resolution (15 μm) (Lanièce *et al*. 1998). Recently, it was applied to anaerobic granules to locate acetate assimilation activities *in situ* in the granule (Collins *et al*. 2005). Although its resolution does not allow detection on the single-cell level, local substrate uptake pattern in the granule can be quantified by beta imaging. The spatial distribution of important phylogenetic groups in the granules was determined by FISH. Finally, the microbial activity distribution determined by the beta imaging and with microsensors was compared with the spatial distribution of the microorganisms.

MATERIALS AND METHODS

Sludge source

Anaerobic granular sludge was collected from a laboratory-scale UASB reactor operated at 35 °C (Satoh *et al*. 2007). The laboratory 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 of 8.2 h. The synthetic medium contained powdered skimmed milk (1,250 mg L^{-1}) as carbon and energy sources, $NaHCO_3$ (1,000 mg L^{-1}), K_2HPO_4 (50 mg L^{-1}), and the mineral solution (Satoh *et al*. 2007). Granule samples were obtained from the upper part of the UASB reactor after 1 year of operation. The water qualities of the effluent during the sampling period were reported in the previous study (Satoh *et al*. 2007).

Microsensor measurements

For microsensor measurements, the granules ca. 2 mm in diameter were selected and positioned using five insect needles in the flow cell reactor (4.0 L) that was filled with the synthetic medium used for the laboratory scale UASB reactor at 35 °C (Satoh *et al*. 2007). 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 N_2, 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 CH_4, H_2, pH and ORP in the granules were measured using microsensors as described by Okabe *et al*. (1999) and Satoh *et al*. (2004). 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 CH_4 were constructed as described by Damgaard & Revsbech (1997). Because all measurements were performed under anoxic conditions, an oxygen-scavenging guard capillary (Damgaard *et al*. 1998) was not applied. A culture of the methane-oxidizing bacterium, *Methylosinus trichosporium* (ATCC 49243), was used as inoculum. The tip diameters of the microsensors were from 50 to 100 μm. Calibration was routinely

performed before and after a measurement by placing a CH_4 microsensor in the synthetic medium for microsensor measurements in a calibration chamber (100 mL) into which CH_4 and N_2 gases were continuously blown at known flow rates at 35 °C. After the sensor signal stabilized, the signal was monitored and the dissolved CH_4 concentration was measured by using the headspace method (Bandara *et al.* 2011). The dissolved CH_4 concentration was changed stepwise by changing the flow rates of CH_4 and N_2 gases. This procedure was repeated over the full range of 0–100% CH_4 saturation. Sensor life span was ca. 2 weeks. H_2 microsensors were constructed as described by Ebert & Brune (1997). 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 H_2 and N_2 gases. The dissolved H_2 concentration in the medium was measured by using the headspace method (Bandara *et al.* 2011). H_2 concentration was changed stepwise by changing the flow rates of H_2 and N_2 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.* (2005). All ORP data reported in this paper were relative to the Ag/AgCl reference electrode. pH microsensors were constructed, calibrated, and used according to the protocol as described by Satoh *et al.* (2004).

Microbial activity calculations

Net volumetric CH_4, H_2 and acid (H^+) production rates ($R(CH_4)$, $R(H_2)$ and $R(H^+)$, respectively) in the granule were estimated from the measured concentration profiles of CH_4, H_2 and pH by using Fick's second law of diffusion as previously described by Santegoeds *et al.* (1999) and Lorenzen *et al.* (1998). For calculation, molecular diffusion coefficients (D_{w25}) of 1.49×10^{-5} $cm^2\ s^{-1}$ for CH_4, 4.50×10^{-5} $cm^2\ s^{-1}$ for H_2, and 9.31×10^{-5} $cm^2\ s^{-1}$ for H^+ in water at 25 °C were used for the calculations (Cussler 1997). Molecular diffusion coefficients in water at 35 °C (D_{w35}) were calculated according to the Stokes-Einstein relationship (Cussler 1997). 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 the previous study (Satoh *et al.* 2007).

Fluorescent *in situ* hybridization

After microsensor measurements, the granule samples were immediately 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 (Okabe *et al.* 1999). After rapid freezing at −21 °C, 20 μm-thick slices were prepared with a cryostat (Reichert-Jung Cryocut 1800, Leica, Bensheim, Germany) and mounted on gelatin-coated slides.

In situ hybridization was performed according to the procedure described by Amann (1995) and Okabe *et al.* (1999). The following 16S and 23S rRNA-targeted oligonucleotide probes were used: EUB338 (Amann *et al.* 1990), EUB338 II (Daims *et al.* 1999), EUB338 III (Daims *et al.* 1999), ARC915 (Raskin *et al.* 1994), SmiSR354 (Ariesyady *et al.* 2007), Synbac824 (Ariesyady *et al.* 2007), and GNSB-941 (Gich *et al.* 2001). The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC). 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.

Radiotracer incubations with granules

Aliquots of 0.5 mL of granule samples were added to 1.5 mL tubes for radiotracer incubations. Sodium [1-^{14}C]propionate (56 mCi/mmol) and D-[U-^{14}C]glucose (320 mCi/mmol) were used as substrate. The organic substrate concentration in each tube ranged from 20 to 100 mM. Incubations were carried out at 35 °C for 8 h; a time-series experiment was achieved by stopping some incubations after 2 and 4 h. Sterilized biomass samples were used as negative controls.

Preparation of granule samples for beta imaging

After incubation, 20 μm-thick slices of the granule samples were prepared as described above. Following washing and drying, the cryosections were covered with a BAS-TR2025 imaging plate (Kobayashi *et al.* 2000). Incorporation of [^{14}C] was quantified by use of a BAS-2500 readout system (Fujifilm Co., Tokyo, Japan) of pixel size 50 × 50 μm. After determination of the original surface of the granule samples in the 2D visualized data, scintillation signals were obtained (as photo-stimulated luminescent (PSL)) from whole

granules. The working principle of the beta microimager is described elsewhere (Lanièce *et al.* 1998).

Chemical analyses

The concentrations of COD were determined according to Standard Methods (APHA *et al.* 2005) using low-range COD reagent vials from Hach Company (Loveland, CO). Volatile fatty acids (VFA) 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 mm pore size membranes (Advantec Co., Ltd., Tokyo, Japan) (Satoh *et al.* 2007). CH_4, H_2 and CO_2 in gaseous samples were determined by gas chromatography (GC-14B; Shimadzu Co.) equipped with a thermal-conductivity detector and a Shincarbon-ST column (Shimadzu Co.) (Satoh *et al.* 2007). ORP and pH were directly determined using an ORP and a pH electrode, respectively.

RESULTS AND DISCUSSION

Spatial distribution of *bacteria* and *Archaea* in granules

FISH was conducted to examine the population abundance and spatial distribution of *Bacteria* and *Archaea* in anaerobic granule samples. Figure 1 is the FISH image of the anaerobic granule using two domain-specific probes: EUB338-mixed probe and ARC915 probe. The result showed that the granule exhibited a layered structure. The outer layer (from surface to 300 μm depth) of the granule was dominated by bacterial cells while the inner layer (below ca. 300 μm from the surface) was occupied mainly by archaeal cells. Similar layered structures of microorganisms in anaerobic granules have been reported in previous studies (Sekiguchi *et al.* 1999; Lanthier *et al.* 2002; Satoh *et al.* 2007). Bacterial and archaeal signals were low in the granule interior (below ca. 400 μm from the surface). The nonstaining center was always observed in the granules analyzed with diameters exceeding about 1,000 μm. This layered structure was repeatedly observed in all of the granular sections analyzed. 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 used in this study (Satoh *et al.* 2003).

FISH results revealed that filamentous cells, which hybridized with the probe GNSB-941 specific for almost all members of the phylum *Chloroflexi*, covered the granule surface (Figure 1(b)). To investigate spatial distribution of syntrophic bacteria, the probe SmiSR354 specific for *Smithella* spp. and the probe Synbac824 specific for *Syntrophobacter* spp. were used. The cells hybridized with the probe SmiSR354 or Synbac824 were detected in the middle layer (at a depth of ca. 100 μm) of the granule (Figure 1(c)). Ariesyady *et al.* (2007) 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. They revealed that *Chloroflexi* and *Smithella* spp. and *Syntrophobacter* spp. were one of the numerically dominant glucose- and propionate-utilizing bacterial groups, respectively (Ariesyady *et al.* 2007). Therefore, it is likely that *Chloroflexi* fermented complex organic compounds (e.g. glucose) in the outermost layer, then *Smithella* spp. and *Syntrophobacter* spp. oxidized VFA (e.g. propionate) into H_2 (and acetate) in a depth of ca. 100 μm, and finally *Archaea* produced CH_4 below ca. 300 μm from the surface.

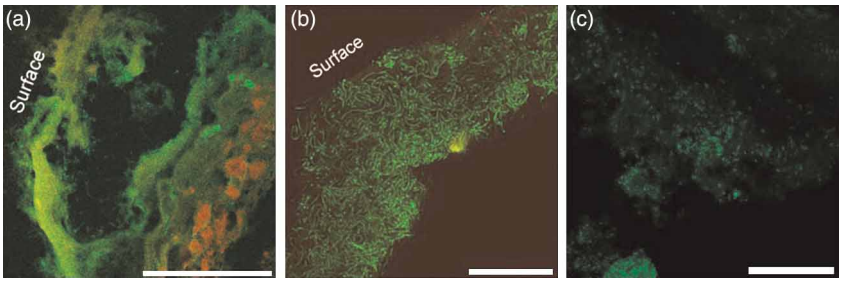

Figure 1 | Confocal laser scanning microscope images showing the *in situ* spatial organization of microorganisms in the anaerobic granules. (a) FISH with FITC-labeled EUB338-mixed probe (green) and TRITC-labeled probe ARC915 (red). (b) FISH with FITC-labeled GNSB-941 probe (green). (c) FISH with FITC-labeled SmiSR354 probe (green). Scale bars indicate 200 μm (a) and 50 μm (b and c).

Concentration profiles and spatial distribution of microbial activities

To confirm this assumption, steady-state concentration profiles of ORP, pH, H_2 and CH_4 in the granules are measured with microsensors (Figure 2). ORP significantly decreased within 200 μm depth (Figure 2(a)). pH moderately decreased within the diffusive boundary layer and 100 μm depth. When H_2 profiles were measured in the medium without $NaHCO_3$, the H_2 profile showed a peak of 18 μM at 100 μm depth and H_2 concentration was under the detection limit (1 μM) below 400 μm from the surface (Figure 2(b)). The addition of 12 mM of $NaHCO_3$ stimulated H_2 consumption activity by hydrogenotrophic methanogens and homoacetogens so that H_2 concentration in the granule was under the detection limit (data not shown). CH_4 concentration gradually increased toward the granule center and finally reached approximately 100 μM (Figure 2(b)). H_2S concentration measured with a H_2S microsensor was below the detection limit (data not shown), due to low sulfate concentration (0.4 mg L^{-1}) in the medium for the microsensor measurements.

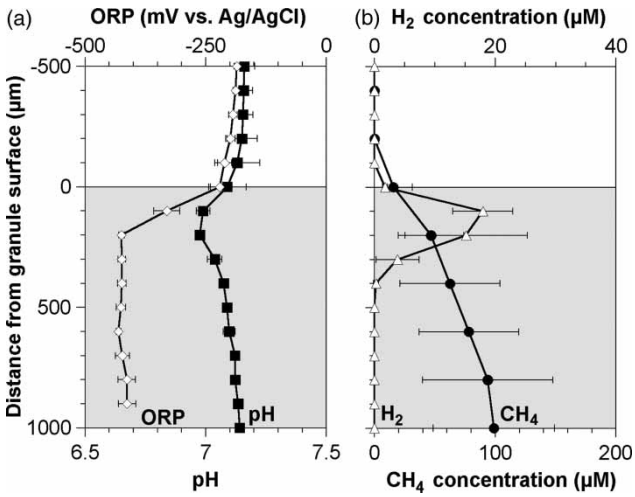

Figure 2 | Concentration profiles of pH and ORP (a), and CH_4 and H_2 (b) in the anaerobic granule. The profiles are average values ($n = 3$) and error bars represent the standard deviations of triplicate measurements. Zero on the vertical axis corresponds to the surface of the granule.

The spatial distributions of $R(CH_4)$, $R(H_2)$ and $R(H^+)$, respectively, were calculated on the basis of the measured concentration profiles (Figure 2) and shown in Figure 3. Figure 3 shows that anaerobic processes occurred in distinctly different layers within the granule. H^+ (acid) production (i.e. fermentation) was found mainly at 100 μm depth with the maximum rate of 0.06 ± 0.02 μmol cm^{-3} h^{-1} and below which acid was consumed (Figure 3(a)). H_2 production (i.e. a syntrophic activity) was detected at a depth from 100 to 200 μm of the granule and below which H_2 was consumed (Figure 3(b)). CH_4 was produced below 200 μm from the surface with the maximum rate of 2.2 ± 3.5 μmol cm^{-3} h^{-1} at a depth of 400 μm (Figure 3(c)). A similar layered structure of the microbial activities was found in methanogenic–sulfidogenic aggregates, in which

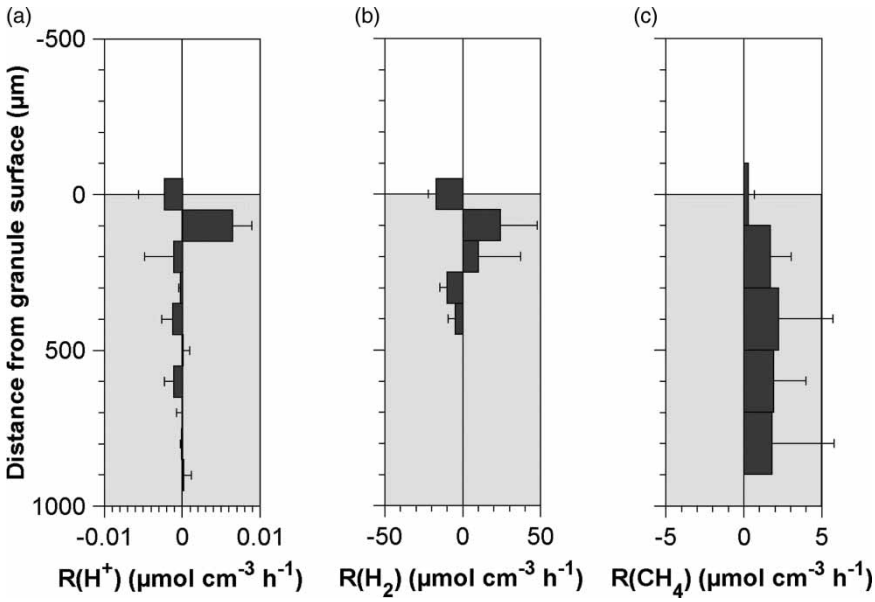

Figure 3 | Spatial distribution and magnitude of the net volumetric production rates of acid (i.e. H^+) (a), H_2 (b) and CH_4 (c). The rates were calculated based on the corresponding concentration profiles shown in Figure 2. Negative values indicate consumption rates. Zero on the vertical axis corresponds to the surface of the granule.

sulfidogenic activity was found in the outer layer and CH_4 production only started from 300 μm onwards inside the aggregate (Santegoeds *et al*. 1999). Although the granules investigated in this study were different from those in the previous study (Satoh *et al*. 2007), the trend in spatial distribution of microbial activities were similar.

As we could not measure the concentration profiles of glucose and VFA with microsensors, substrate (i.e. glucose and propionate) uptake patterns in the granules were investigated using beta imaging (Figure 4) and radioactivity was quantitatively analyzed (Figure 5). Beta imaging revealed the localized incorporation of glucose and propionate in the granule. The uptake of [^{14}C] was highest at 50 μm depth and decreased gradually toward the granule center in glucose incubation. Signals were very low below 600 μm depth. In contrast, tracer was incorporated from the surface to 500 μm depth with a peak at 200 μm depth in propionate incubation. The uptake of [^{14}C] was higher in glucose incubation than in propionate incubation.

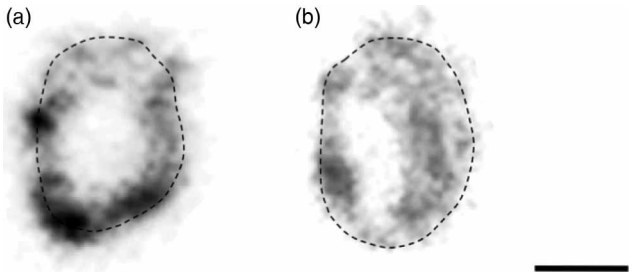

Figure 4 | Two-dimensional distribution of [^{14}C]glucose (a) and [^{14}C]propionate (b) incorporation in thin section of the granules as visualized by beta imaging. Signal intensity per pixel (50 by 50 μm) is indicated by black. Dotted line indicates surface of the granule. Scale bar indicates 500 μm.

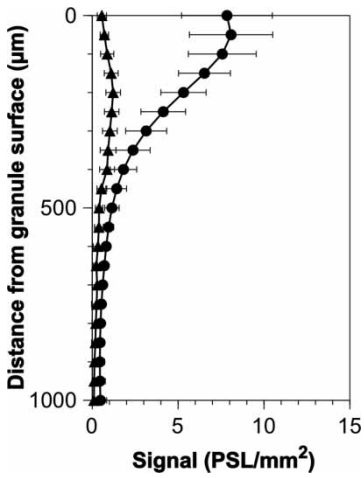

Figure 5 | Vertical distribution of local scintillation signals in granules incubated with sodium [1-^{14}C]propionate (▲) and D-[U-^{14}C]glucose (•) determined from beta microimaging data (Figure 4). The profiles are average values ($n = 6$) and error bars represent the standard deviations.

CONCLUSIONS

Direct comparison of the microbial activities analyzed by microsensor technique and beta imaging with spatial distribution of the microorganisms analyzed by FISH demonstrated that the spatial distributions of the microorganisms and their activities in the single anaerobic granules were characterized by a distinct layered structure. *Chloroflexi* fermented glucose in the outermost layer, *Smithella* spp. contributed to propionate oxidation and H_2 production at a depth from 100 to 200 μm, and *Archaea* produced CH_4 below ca. 300 μm from the surface. Thus, the combined use of FISH, beta imaging and microsensors clearly demonstrated the comprehensive pathway of the anaerobic conversion (from degradation of complex organic compounds to CH_4 production) by each different trophic group within single anaerobic granules.

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