Aggregation Ability of Three Phylogenetically Distant Anammox Bacterial Species

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ABSTRACT

Anaerobic ammonium-oxidizing (anammox) bacteria are well known for their aggregation ability. However, very little is known about cell surface physicochemical properties of anammox bacteria and thus their aggregation abilities have not been quantitatively evaluated yet. Here, we investigated the aggregation abilities of three different anammox bacterial species: “*Candidatus Brocadia sinica*”, “*Ca. Jettenia caeni*” and “*Ca. Brocadia sapporoensis*”. Planktonic free-living enrichment cultures of these three anammox species were harvested in membrane bioreactors (MBRs). The physicochemical properties (*e.g.*, contact angle, zeta potential, and surface thermodynamics) were analyzed for these anammox bacterial species and used in the extended DLVO theory to understand the force-distance relationship. In addition, their extracellular polymeric substances (EPSs) were characterized by X-ray photoelectron spectroscopy and nuclear magnetic resonance. The results revealed that the “*Ca. B. sinica*” cells have the most hydrophobic surface and less hydrophilic functional groups in EPS than other anammox strains, suggesting better aggregation capability. Furthermore, aggregate formation and anammox bacterial populations were monitored when planktonic free-living cells were cultured in up-flow column reactors under the same conditions. Rapid development of microbial aggregates was observed with the anammox bacterial population shifts to a dominance of “*Ca. B. sinica*” in all three reactors. The dominance of “*Ca. B. sinica*” could be explained by its better aggregation ability and the superior growth kinetic properties (higher growth rate and affinity to nitrite). The superior aggregation ability of “*Ca. B. sinica*” indicates significant advantages (efficient and rapid start-up of anammox reactors due to better biomass retention as granules and consequently stable performance) in wastewater treatment application.

KEYWORDS: Anammox bacteria, aggregation ability, cell surface physicochemical properties, extracellular polymeric substances

1. INTRODUCTION

Anaerobic ammonium-oxidizing (anammox) bacteria are chemolithoautotrophs with anammoxosome, a unique organelle in which energy is generated by oxidizing ammonium into nitrogen gas with nitrite as an electron acceptor (Jetten et al., 2005). Anammox bacteria are affiliated with the phylum *Planctomycetes*, and five candidatus genera (*Brocadia*, *Kuenenia*, *Jettenia*, *Scalindua*, and *Anammoxoglobus*) and more than 25 species have been tentatively proposed for anammox taxonomic group (Ali and Okabe, 2015; Awata et al., 2013; Kartal et al., 2007; Oshiki et al., 2011). Anammox activities and corresponding bacteria have been detected in various natural and human-made ecosystems (Sonthiphand et al., 2014). Most anammox bacteria discovered from anoxic marine systems exclusively belong to the genus “*Candidatus Scalindua*” with a niche characterized by thriving in high salt (2.0 – 3.6 %) and low temperature (4 - 20°C) environments (Awata et al., 2013; Schmid et al., 2007; Villanueva et al., 2014). In contrast, other four genera were mostly detected from engineered and freshwater ecosystems (Egli et al., 2001; Hu et al., 2011; Schmid et al., 2000; Sonthiphand et al., 2014; Strous et al., 1999). There is still no pure culture available for the anammox species. Therefore, various enrichment cultures of anammox species (>90% purity) have been used to study their ecophysiology and biochemistry (Ali et al., 2015a; Awata et al., 2015; T Lotti et al., 2014; Oshiki et al., 2016a, 2011).

Population shifts from one anammox genera or species to another were often demonstrated in many studies (*i.e.*, “*Ca. B. fulgida*” to “*Ca. B. sp.40*” (Park et al., 2010), “*Ca. B. fulgida*” to “*Ca. K. stuttgartiensis*” (Park et al., 2015), “*Ca. B. sp.40*” to “*Ca. K. stuttgartiensis*” (van der Star et al., 2008), “*Ca. B. anammoxidians*” to “*Ca.*

Anammoxoglobus propionicus” (Kartal et al., 2007)). Population dynamics of anammox bacterial species were systematically investigated in nitrite-limited bioreactors recently (Zhang et al., 2017a). A population shift from “*Ca. B. sp.40*” to “*Ca. K. stuttgartiensis*” was directly attributed to limiting substrate concentration (nitrite), where the authors proposed “*Ca. Brocadia*” as the growth rate (μ) strategist and “*Ca. Kuenenia*” as the affinity (K) strategist (van der Star et al., 2008). Their findings likely explain the natural distributions of anammox bacteria in freshwater ecosystems. Population shifts in anammox communities are affected by several selective pressures including seeding inoculum (Osaka et al., 2012; Park et al., 2010), tolerance to salinity (Awata et al., 2015; Sonthiphand et al., 2014), presence of organic acids an electron donor for nitrate reduction (Kartal et al., 2008), and different affinity with ammonium, nitrite and/or dissolved oxygen (Park et al., 2015; van der Star et al., 2008). These studies indicated the presence of specific niche for each anammox genera or species. Nevertheless, the niche differentiation of anammox bacteria is still largely unknown.

It has been well recognized that anammox bacteria tend to form microbial aggregates (Chen et al., 2013; Egli et al., 2001) and biofilms (Innerebner et al., 2007). Extracellular polymeric substances (EPS) released by bacteria have been considered to play a vital role in bacterial cell aggregation (Flemming and Wingender, 2010; Liu et al., 2010). Furthermore, the composition of EPS excreted by “*Ca. B. fulgida*” dominated biomass was analyzed, and it was found that hydrophobic interaction was a main force for formation of aggregates (Hou et al., 2015). However, there is limited information regarding the cell surface physicochemical properties and aggregation ability of various other anammox bacteria.

In this study, we first developed planktonic free-living enrichment cultures of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*” using membrane bioreactors (MBRs). The physicochemical properties (*i.e.*, contact angle, zeta potential, and surface thermodynamics) of each enriched cell were analyzed to evaluate their aggregation abilities. In addition, the

chemical structure and composition of EPS were characterized using X-ray photoelectron spectroscopy (XPS) and nuclear magnetic resonance (NMR) technique. Furthermore, each planktonic free-living enrichment culture was incubated in up-flow glass column reactors equipped with a thin glass filter to retain only aggregated bacterial cells. We monitored aggregate formation and population dynamics of anammox bacteria during the cultivation to verify their aggregation abilities.

2. MATERIALS AND METHODS

2.1. Biomass origin.

Highly enriched planktonic free-living cultures of “*Ca. B. sinica*” (Oshiki et al., 2013), “*Ca. J. caeni*” (Ali et al., 2015a) and “*Ca. B. sapporoensis*” (Narita et al., 2017) were harvested in MBRs equipped with a hollow-fiber membrane unit composed of 300 polyethylene tubes (pore size, 0.1 μm ; tube diameter, 1 mm; length, 70 mm) as previously described. These MBRs were fed with inorganic nutrient medium at nitrogen loading rates (NLRs) of 0.48–1.02 $\text{kg-N m}^{-3} \text{d}^{-1}$ at 37°C. In these MBRs, the anammox bacterial cells were kept in free-living planktonic by stirring at 150 rpm. Anoxic condition was maintained by continuous sparging of mixed gas (5% CO_2 and balance Argon gas) into the cell cultures at a flow rate of 10 mL min^{-1} . pH was not controlled throughout the cultivation but it was always within a range of 7.0–8.0.

2.2. Physicochemical analysis of bacterial cells.

Planktonic anammox bacterial cells harvested from the MBRs were filtered on 0.45- μm acetate cellulose membranes and washed three times with deionized water. A piece of membrane was cut off and air-dried for 10 min. The sludge contact angles against water, glycerol, and formamide were measured using the sessile drop technique (Khan et al., 2011;

Liu et al., 2010). The average contact angles were obtained from ten measurements. Anammox bacterial cells were diluted in 0.1 M NaCl solution to approximately 0.1 g-protein L⁻¹, and pH of the bacterial cell suspension was adjusted to 7.6 (if required) with 0.1M HCl and 0.1M NaOH. Thereafter, zeta potential was measured using a Zetasize Nano ZS Instrument (Malvern. Inc., USA). The average values were calculated based on six replicate measurements. The average contact angle and zeta potential were then used to calculate the surface free energy for aggregation for the three enriched bacterial cultures. Thus, the interaction energy as a function of distance between the bacterial cells was calculated according to the extended DLVO theory (Liu et al., 2010; van Oss, 2008). The total energy of interaction (W_T) can be obtained by addition of the electric double layer (W_R), van der Waals energies (W_A) and the acid-base interaction (W_{AB}). The total interaction energies were expressed as a function of the separation distance H . The detailed calculations were presented in the Supporting Information.

2.3. EPS extraction and composition analysis.

EPS was extracted from each MBR planktonic culture as described previously (Tan et al., 2014), with some modifications. Briefly, 50-ml of planktonic anammox bacterial cell cultures were centrifuged at 12,000×g for 10 min. and the pellets were subjected to freeze-drying for 24 h. The weight of dried biomass was recorded. Then, the samples were re-suspended in 5 mL of 0.9% NaCl (w/v) solution with 0.6% formaldehyde (v/v) for 1 h at 4°C. Subsequently, 2 mL of 1 M NaOH was added to the suspension and stored for 3h at 4°C before collecting EPS at 12,000×g for 15 min at 4°C. The polysaccharide component of EPS was measured using the phenol-sulfuric acid assay with glucose as the standard (DuBois et al., 1956), whereas the protein component was quantified using the Lowry method with DC protein assay kit (Bio-Rad; CA, USA), with bovine serum albumin as the standard.

2.4. X-ray photoelectron spectroscopy.

The XPS spectra were obtained by X-Ray Photoelectron Spectrometer (JEOL JPS-9010MX, Japan) to determine the elemental composition and functional groups in the EPS extracted from the bacterial cells. A broad survey scan (20.0 eV pass energy) was used to determine the major elemental component, and a high-resolution scan (70.0 eV pass energy) was conducted for component speciation.

2.5. Nuclear magnetic resonance (NMR) analysis.

The lyophilised EPS samples were dissolved in 600 μL of deuterated water (D_2O), and then 550 μL of the solution was transferred to 5 mm NMR tubes. NMR spectra were recorded using Bruker 950 MHz AVANAC III NMR spectrometer equipped with Bruker CP TCI multinuclear CryoProbe (BrukerBioSpin, Rheinstetten, Germany). The ^1H NMR spectra were recorded by collecting 4k scans with a recycle delay time of 5 s using excitation sculpting pulse sequence (zgpg30) program from Bruker pulse library. Chemical shifts were adjusted using 3-Trimethylsilylpropane sulfonic acid (DSS, 20 mM) as internal chemical shift reference. Chemical shifts were determined relative to 3-Trimethylsilylpropane sulfonic acid (DSS, 20 mM). The free induction decay (FID) data were collected with a spectral width of 16 ppm into 64 k data points. The FID signals were amplified by an exponential line-broadening factor of 1 Hz before Fourier transformation. Bruker Topspin 2.1 software (Bruker BioSpin, Rheinstetten, Germany) was used to collect and analyze the data.

2.6. Cultivation in column reactors.

Each planktonic free-living anammox bacterial cultures was harvested from the MBRs and then inoculated into up-flow glass column reactors (60-mL, FujiRika, Osaka, Japan) equipped with glass filter (pore size 100 – 200 μm) (**Fig. S1**). The initial biomass concentration was adjusted to 1.25 g-protein L^{-1} in each column reactor. The column reactors inoculated with the enriched culture of “*Ca. B. sinica*”, “*Ca. J. caeni*” or “*Ca. B. sapporoensis*” hereafter referred to as Reactor 1 (R1), Reactor 2 (R2) and Reactor 3 (R3),

respectively. Particle size distributions of bacterial cultures were measured by using MasterSizer (Malvern Inc., USA). These column reactors were continuously fed with synthetic medium and operated for three months at 37°C. The synthetic medium used in the current study contained NH_4^+ (variable) mM, NO_2^- (variable) mM, and inorganic nutrient medium (mg/L): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (9.0), $\text{EDTA} \cdot 4\text{Na}$ (5.0), NaCl (1.0), KCl (1.4), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), NaHCO_3 (84), KH_2PO_4 (54) as described elsewhere (van de Graaf et al., 1995). Initially, the concentrations of NH_4^+ and NO_2^- were set at 2.5 mM each and gradually increased to 5.0, 7.5, 10.0, 15.0 and 20.0 mM, which resulted in nitrogen loading rates (NLRs) of 2.3, 4.5, 6.6, 8.7, 13.1 and 17.5 $\text{kg-N m}^{-3} \text{d}^{-1}$, respectively. Hydraulic retention time (HRT) was set at 0.8 h in all reactors. Concentrations of NH_4^+ , NO_2^- and NO_3^- in the influent and effluent of the column reactors during the operation were determined using ion chromatography (IC-2010, TOSOH, Tokyo, Japan) equipped with TSKgel IC-Anion HS and TSKgel IC-Cation columns (TOSOH) after filtration using 0.45- μm membrane filters (Advantec, Tokyo, Japan).

2.7. Quantitative PCR assays.

The specific TaqMan® qPCR assays were used to quantify the 16S rRNA gene copy numbers of each anammox bacterial strain “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*”. The TaqMan® qPCR assays with specific primer sets and TaqMan® probes (**Table S1**) were designed previously (Narita et al., 2017; Zhang et al., 2017a). The 16S rRNA gene copy numbers assumed, reflecting the number of actual anammox bacterial cells in the reactor. Biomass samples were collected from the column reactors on a monthly basis, and the genomic DNA was extracted using FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) according to manufacturer’s instructions. The composition of each PCR mixture (20 μL) was as follows: Premix Ex Taq™ (2X) 10 μL , forward primer (10 μM) 0.4 μL , reverse primer (10 μM) 0.4 μL , TaqMan® probe (10 μM) 0.8 μL , ROX™ Reference Dye II (50X) 0.4

μL, DNA template (sample DNA or standard) 2 μL, and dH₂O 6 μL. The standard curves for quantification of the three anammox bacterial species were prepared by 10-fold serial dilutions (10⁷ to 10¹ copy numbers per μL) of the respective plasmid DNA carrying the target 16S rRNA gene. All PCR amplifications and detections were conducted in MicroAmp Optical 96-well reaction plates (Applied Biosystems, California, USA) using ABI prism 7500 Real-time PCR system (Applied Biosystems, California, USA) with the following PCR conditions: initial denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, annealing/extension at 60°C for 34 s.

3. RESULTS

3.1. Bacterial surface properties.

To characterize the surface properties of anammox bacterial cells, the contact angles against water, glycerol, and formamide were measured (**Table S2**). The contact angles of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*” against water were 79.5°, 72.5°, and 60.8°, respectively, demonstrating that *Ca. B. sinica* cells have the most hydrophobic surface, whereas *Ca. B. sapporoensis* cells have the most hydrophilic surface. The zeta potentials were also measured to evaluate surface electronegativity (**Table S2**). The zeta potentials of the three enriched anammox bacterial species were not significantly different.

The contact angle and zeta potential were then used to calculate the surface free energy for aggregation as described in the Supporting Information (**Table 1**). The interfacial free energy (ΔG_{adh}) of “*Ca. B. sinica*” cells was -26.1 mJ m^{-2} , which was lower than those of other anammox bacterial species, suggesting that the surface of “*Ca. B. sinica*” cells had more free energy available for microbial adhesion. Compared to $\Delta G_{\text{adh}}^{\text{LW}}$ (the Lifshitz-van der Waals interactions), $\Delta G_{\text{adh}}^{\text{AB}}$ (the Lewis acid-base interactions) had a higher absolute value

and therefore was a significant contributor to $\Delta G_{\text{adh}}^{\text{total}}$ of anammox biomass, namely the Lewis acid-base interactions played a crucial role in the aggregation process.

The degree of cell-cell adhesion, initial aggregation step, was qualitatively characterized using the extended DLVO theory. The different interaction energies (i.e. W_T , W_R , W_A and W_{AB}) were calculated for all three anammox bacterial strains as a function of distance between the bacterial cells (**Fig. S2**). The results indicated that all anammox bacterial cells had no obvious potential energy barrier. W_A was active only for a short distance, while the values of W_{AB} were high for relatively long distances and negative for all three anammox bacterial strains, implying that they attracted each other mainly through hydrophobic interaction.

3.2. Characterization of EPS.

Further EPS analysis was required to explain the detailed mechanism of hydrophobic interaction. The bacterial EPS was mainly composed of protein and polysaccharide, with minor quantities of extracellular DNA, humic acids and lipids (**Fig. 1**).

The elemental forms of EPS carbon were determined by narrow scan XPS C 1s spectra. The XPS spectrum was analyzed by using an open source peak fitting software (XPS Peak 4.1). The element C primarily comprised of four forms: C-(C/H), C-OH, C=O and O-C=O (**Fig. 2**). In the EPS excreted by “*Ca. B. sinica*” cells, the element C form C-(C/H) (carbon chain and hydrocarbyl) accounted for 62.8% (**Table 2**), comprising the hydrophobic components of EPS. This value was significantly higher than those of “*Ca. J. caeni*” (43.9%) and “*Ca. B. sapporoensis*” (52.0%). Similarly, the hydrophilic carbon components in EPS (i.e. C-OH, C=O and O-C=O) accounted for 37.2, 56.1 and 48.0% for “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*”, respectively. This observation was consistent with the

results of surface thermodynamics, confirming the high hydrophobic nature of “*Ca. B. sinica*” cells.

3.3. ¹H NMR fingerprint of EPS.

The ¹H solution-state NMR spectrum of EPS was determined at 950 MHz (**Fig. 3**). Chemical shift assignment and interpretation of different peaks were carried out based on existing data reported in the literature (Gonzalez-Gil et al., 2015; Seviour et al., 2010; Simpson et al., 2011). Broadly, peaks observed between 0.5 – 2.2 ppm, 2.2 – 5.3 ppm, and 6.2 – 8.5 ppm chemical shift were characterized as aliphatic, sugar and/or carbohydrates, an aldehyde and/or aromatic substances groups, respectively. Within the aliphatic group, the peaks between 0.5 – 1.0 ppm could be assigned to methyl groups mainly from peptides. The peak regions between 1.0 – 1.5 ppm and 1.5 – 2.2 ppm correspond to CH₂γ to COOH and β to COOH, respectively. Peaks between 2.2 – 3.6 ppm, 3.6 – 4.0 ppm, 4.0 – 4.6 ppm and 5.0 – 5.3 ppm were assigned to carbohydrate, sugar ring protons, alginate, and anomeric protons (carbohydrate), respectively. The peak broadening in the sugar region suggests that the EPS samples of “*Ca. B. sinica*” and “*Ca. J. caeni*” contained large molecules such as alginate-like polysaccharides (Gonzalez-Gil et al., 2015). Gray dotted line in **Fig. 3** shows that “*Ca. B. sinica*” and “*Ca. J. caeni*” contained more alginate-like polysaccharides than “*Ca. B. sapporoensis*”. In aldehyde and/or aromatic substances group, the peak regions were assigned as 6.2 – 6.8 ppm (aromatic amino acid side chains), 6.8 – 7.4 ppm (substitute aromatics) and 7.4 – 8.5 ppm (aldehydes). The ¹H NMR peak regions were integrated and normalized for relative quantification (**Fig. 4**), which revealed that alginate content was slightly higher in “*Ca. B. sinica*” as compared to the other anammox species evaluated.

3.4. Performance of the column reactors and granule development.

Three up-flow glass column reactors, R1, R2, and R3, were inoculated with MBR enrichment planktonic cultures of “*Ca. B. sinica*”, “*Ca. J. caeni*”, and “*Ca. B. sapporoensis*”,

respectively to verify their aggregation ability. These column reactors were continuously supplied with the inorganic nutrient medium at NLR of $2.3 \text{ kg-N m}^{-3} \text{ d}^{-1}$ and gradually increased to about $17.5 \text{ kg-N m}^{-3} \text{ d}^{-1}$ within 3 months. The average nitrogen removal rates (NRRs) over the 3-month period were $8.4 (1.3 - 13.5)$, $8.4 (0.5 - 13.7)$ and $4.7 (1.5 - 7.8) \text{ kg-N m}^{-3} \text{ d}^{-1}$ for R1, R2, and R3, respectively (**Fig. 5**). Overall the performance of R1 and R2 was slightly better than R3. The nitrite concentration was $< 10 \text{ mg-N L}^{-1}$ most of the time during the operation. The calculated stoichiometric ratios of all three reactors were close to the theoretical stoichiometric ratios of anammox reaction (i.e., 1.15 and 0.16 for removed ΔNO_2^- / removed ΔNH_4^+ and produced ΔNO_3^- / removed ΔNH_4^+ , respectively) (Lotti et al., 2014) indicating anammox was the primary nitrogen removal process in these reactors. The initial biomass concentration and the average size of the anammox bacterial aggregates were $1.25 \text{ g-protein L}^{-1}$ and $\sim 50 \text{ }\mu\text{m}$, respectively. The bacterial aggregate size in the three reactors increased during the operation (**Fig. S3**), and ultimately at the end of reactor operation microbial aggregate size grew to 2–5 mm (**Fig. S4**). The microbial community in the aggregates were monitored by measuring 16S rRNA gene copy number as shown below.

3.5. Population dynamics in the column reactors.

The MBR enrichment cultures that were inoculated into the column reactors (R1, R2, and R3) were dominated by “*Ca. B. sinica*” (91.4%), “*Ca. J. caeni*” (75.8%) and “*Ca. B. sapporoensis*” (98.1%), respectively (**Fig. 6**). The “*Ca. J. caeni*” culture was less enriched as compared to the other anammox cultures. It should be noted that the EPS of “*Ca. J. caeni*” may contain the EPS excreted by other bacteria, which should be taken into consideration for meaningful comparison. In the reactor R1, the relative abundance of “*Ca. B. sinica*” increased from 91.4 to 99.8% during the operation, whereas the anammox bacterial populations shifts from “*Ca. J. caeni*” to “*Ca. B. sinica*” and “*Ca. B. sapporoensis*” to “*Ca. B. sinica*” were observed in the reactors R2 and R3, respectively. The lower NRR in R3 could

be attributed to the population transition of “*Ca. B. sapporoensis*” to “*Ca. B. sinica*” and it took a while by “*Ca. B. sinica*” to grow in a critical mass to achieve nitrogen removal rates.

4. DISCUSSION

Besides its biogeochemical significance, the anammox process has attracted a great deal of attention as an energy-efficient and environment-friendly wastewater treatment process. In biological wastewater treatment systems, bacterial cell aggregation is essential for efficient liquid/solid separation and biomass retention. In anammox-based nitrogen removal systems, biomass retention is the key to successful start-up and stable operation of the systems due to relatively slow growth rates of anammox bacteria. Besides, granular sludge demonstrated quicker recovery after starvation and/or chemical inhibition (Ali et al., 2015b; Lotti et al., 2014). The formation of anaerobic granules is considered to be a multi-step process, and both physicochemical and biological parameters are involved complicatedly.

The interactions between bacterial cells include, but not limited to, repulsive electrostatic forces, attractive van der Waals forces, and hydration interaction (van Oss, 1995). According to a thermodynamics theory, if an adsorption process could lead to the decrease in the system free energy, adsorption will occur spontaneously (Tanford, 1979; Van Oss et al., 1988). Hence, to interpret the role of physicochemical forces in microbial adhesion process, some thermodynamic models have been developed (Van Oss et al., 1988). It was previously reported that when bacterial surfaces are strongly hydrophobic, irreversible adhesion will occur (Hou et al., 2015; Krasowska and Sigler, 2014; van Oss, 1995). This implies that hydrophobicity (or hydrophilicity) of the bacterial surfaces is a key factor involved in aggregation (Khan et al., 2011). Increasing the hydrophobicity of cell surfaces would lead to a decrease in the excess Gibbs free energy of the surface, which in turn promotes cell-to-cell interaction and further serves as a driving force for cell self-separation

from bulk liquid. Previously, it suggested that the hydrophobicity of bacterial surface plays a crucial role in the commencement of both aerobic and anaerobic granulation (Tay et al., 2001, 2000). The results in this study clearly demonstrated that “*Ca. B. sinica*” cells had better aggregation ability due to the hydrophobic surface.

The significance of hydrophobic interaction on all three anammox bacterial strains was verified by extended DLVO approach. The extended DLVO theory accounts for van der Waals, electrostatic, and acid–base interaction energies between bacterial cells as a function of their separation. For all types of anammox culture, electric double layer (W_R) was not so influential due to its small absolute value. Further, according to the zeta potentials (**Table S2**), the three enriched anammox bacterial cells had similar electronegative functional groups. Van der Waals interaction (W_A) plays a role when the distance between two microbial cells is short enough (less than 0.1 nm). However, as the distance increases, the dominant role turns out to be hydrophobic interactions (W_{AB}). Hydrophobic interactions have the same range as, but are about an order of magnitude stronger than, van der Waals interactions (Israelachvili, 2011). In a previous study, the hydrophobic interaction was reported to be the most significant long-range force in the formation of “*Ca. B. fulgida*”-dominated sludge aggregation (Hou et al., 2015). As a comparison of the hydrophobic interactions among three anammox strains, the interfacial free energy (ΔG_{adh}^{AB}) of “*Ca. B. sinica*” cells had a higher absolute value, and the acid-base interaction energy (W_{AB}) was negative and had longer separation distance as compared to other two strains. This indicates that “*Ca. B. sinica*” cells had stronger hydrophobic interaction than other anammox cells.

Bacterial aggregation is not only a surface chemistry process, but many unidentified biological components and factors are involved, which precludes one from building a purely physical model. Alginate-like exopolysaccharides were proposed to be responsible for maintaining the gel-like structure of aerobic (Lin et al., 2013) and anaerobic granules

(Gonzalez-Gil et al., 2015). Previous report indicated conjugation of alginate with calcium and/or magnesium could further enhance the hydrophobicity of modified alginate (Yao et al., 2010). Calcium was commonly found in anammox granules in the form of mineral and apatite (Lin et al., 2013). In this study, we found that “*Ca. B. sinica*” excreted more alginate-like compounds than other anammox bacteria, which resulted in higher aggregation ability of “*Ca. B. sinica*”.

The geographic distributions of anammox bacteria indicate that they likely have species-specific habitats (Sonthiphand et al., 2014). Growth kinetics are one of essential factors for deciphering the niche differentiation of anammox bacteria. The physiological characteristics and kinetic parameters of various anammox bacterial species have been determined and summarized (Lotti et al., 2015; Narita et al., 2017; Oshiki et al., 2016b; Zhang et al., 2017b). Recently, the μ_{max} values were reevaluated using planktonic free-living enrichment cell cultures under nitrite-limited conditions and ascertained that they could grow much faster than we thought previously. For example, the μ_{max} values of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*” were reported to be 0.33 d⁻¹, 0.18 d⁻¹, and 0.20 d⁻¹, respectively (Narita et al., 2017; Zhang et al., 2017b). In addition, the half-saturation constant (K_S) for nitrite was determined to be 34 ± 21 μ M for “*Ca. B. sinica*”, 35.6 ± 0.92 μ M for “*Ca. J. caeni*”, and 5 ± 2.5 μ M for “*Ca. B. sapporoensis*”, respectively. Based on these kinetic parameters, the Monod growth curves were drawn (**Fig. S5**). “*Ca. B. sinica*”, which has the highest μ_{max} , could outcompete the other anammox species at a nitrite concentration of > 17–30 μ M, only below which “*Ca. B. sapporoensis*”, which has the highest affinity to NO₂⁻ (lowest K_S) among the three species, would overgrow. “*Ca. J. caeni*” could be outcompeted by other two species under any nitrite concentration. However, it is emphasized that these growth kinetics parameters were derived from free living planktonic cultures, which has no substrate gradients, in contrast to the aggregated cell cultures.

It is noted that anammox bacterial density exhibited the similar increasing trend as the NRRs only in R1, which was dominant by “*Ca. B. sinica*” throughout the operation period. However, anammox bacterial densities were not correlated with the NRRs in other reactors, R2 and R3. The population transition from one dominant anammox bacterium, “*Ca. J caeni*” (R2) and *Ca. B. Sapporoensis* (R3), to “*Ca. B. sinica*” might have caused the poor correlation between the bacterial densities and NRRs. There could also be a natural substrate gradients in microbial aggregated which could cause substrate limitation in the core of microbial aggregates as previously noted anammox (Ali et al., 2015c; Okabe et al., 2011). Substrate limitation and elevated pH in the core of microbial aggregate might reduce the overall bacterial activity of aggregated cells. Also, as evidenced in **Fig S4**, the microbial aggregates accumulate inorganic mineral (i.e. apatite), which could result in lesser specific activities, though the accumulation of apatite increases the mechanical property of anammox granules (Y M Lin et al., 2013).

In the current study, the EPS was extracted from the highly enriched planktonic anammox cultures that were harvested from the MBR operating under the similar steady-state condition, and then were characterized in detail to determine their aggregation potential. To verify the aggregation ability, the planktonic free-living anammox bacterial cells were inoculated and cultured in up-flow column reactors equipped with a glass filter at the outlet port. The role of the glass filter was to preferentially retain aggregated bacterial cells in the column reactor. In all the three column reactors, “*Ca. B. sinica*” dominated (**Fig. 7**) after three-month operation due to its high aggregation ability and growth rate. Therefore, it was not worthwhile characterizing EPS during the up-flow column reactor operation since EPS could be excreted by mixed populations of anammox bacterial strains. Though, it could be very interesting to compare the EPS composition between MBR planktonic cultures and aggregated biomass in the column reactors in the future study.

The results of this study can explain the previous reports showing that the anammox populations in granular biomass-based full-scale wastewater treatment systems were exclusively dominated by “*Ca. B. sinica*” (Speth et al., 2016; Suto et al., 2017). The granular sludge has enormous advantages over planktonic and biofilm biomass because of high biomass density, smaller footprint, efficient biomass separation, high tolerance to chemicals and oxygen exposure, and easy inoculation to new reactors (*i.e.*, efficient start-up). The granular sludge-based anammox processes have been already applied in full-scale treatment plants around the world (more than 120 installations) (Ali and Okabe, 2015; Lackner et al., 2014).

5. CONCLUSIONS

- The aggregation abilities of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*” were investigated based on the analyses of physicochemical properties of cell surface and EPS composition.
- “*Ca. B. sinica*” cells had the most hydrophobic cell surface and less hydrophilic functional groups in EPS, which indicated the highest aggregation ability among the three anammox species tested.
- “*Ca. B. sinica*” dominated in all three column reactors due to superior aggregation ability and growth kinetics.
- There are apparently numerous advantages in selection of “*Ca. B. sinica*” as an inoculum of anammox-based nitrogen removal systems including efficient retention of aggregated biomass and consequently the stable performance of the system.

ACKNOWLEDGEMENTS

This research financially supported by Nagase Science and Technology Foundation and Institute for Fermentation, Osaka (IFO), which were granted to Satoshi Okabe, and by Competitive Research Grant (CRG_R2_13_SAIK_KAUST_1) from King Abdullah University of Science and Technology (KAUST). Lei Zhang was supported partly by the Monbukagakusho Honors Scholarship for Privately Financed International Students offered by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. When Muhammad Ali was a Post-Doctoral Fellow at Hokkaido University, Sapporo Japan, he was supported by Graduate School of Engineering, Hokkaido University, Japan.

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TABLES:

Table 1: Interfacial free energy between the bacterial cell surface and water for the three enriched anammox bacterial cultures.

Anammox bacterial species	Interfacial free energy components [mJ m ⁻²]		
	$\Delta G_{\text{adh}}^{\text{LW}}$	$\Delta G_{\text{adh}}^{\text{AB}}$	$\Delta G_{\text{adh}}^{\text{total}}$
<i>Ca. B. sinica</i>	-1.0	-25.1	-26.1
<i>Ca. J. caeni</i>	-5.2	-11.2	-16.4
<i>Ca. B. sapporoensis</i>	-7.0	-3.8	-10.8

Table 2: Quantitative analysis of different element C forms in EPS derived from XPS narrow scan spectra.

Anammox bacterial species	C-(C/H)	C-OH	C=O	O-C=O
<i>Ca. B. sinica</i>	62.8%	26.6%	10.6%	0.0%
<i>Ca. J. caeni</i>	43.9%	19.4%	24.2%	12.4%
<i>Ca. B. sapporoensis</i>	52.0%	26.9%	19.0%	2.1%

FIGURE CAPTIONS:

Figure 1. Protein and carbohydrate fraction of extracellular polymeric substances released by enriched anammox cultures of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*”.

Figure 2. High-resolution X-ray photoelectron spectroscopy spectra (C 1s) of EPS harvested from the MBR enrichment cultures of “*Ca. B. sinica*” (**A**), “*Ca. J. caeni*” (**B**) and “*Ca. B. sapporoensis*” (**C**), respectively.

Figure 3. ¹H solution-state NMR spectrum at 950 MHz of EPS harvested from the MBR enrichment cultures of “*Ca. B. sinica*” (**A**), “*Ca. J. caeni*” (**B**), and “*Ca. B. sapporoensis*” (**C**), respectively.

Figure 4: Fraction assignments and integration of EPS for the difference anammox cultures (based on the spectrum of proton NMR, **Fig. 3**).

Figure 5: Time course of nitrogen removal rates (NRRs) in the three column reactors inoculated with MBR enrichment cultures of “*Ca. B. sinica*” (Reactor 1), “*Ca. J. caeni*” (Reactor 2), and “*Ca. B. sapporoensis*” (Reactor 3), respectively. These reactors were operated under the same conditions at 37°C for three months.

Figure 6: Time course variation of copy numbers of 16S rRNA gene and the relative abundance (%) of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. B. sapporoensis*” in Reactor 1 (**A**, **B**), Reactor 2 (**C**, **D**) and Reactor 3 (**E**, **F**). Error bars indicate the standard deviation of triplicate PCR reactions.

Figure 1

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Figure 4

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