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Physical enrichment of uncultured *Accumulibacter* and *Nitrospira* from activated sludge by unlabeled cell sorting technique

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It is important to understand the ecology and physiology of microbes in activated sludge of wastewater treatment plants. Recently, molecular based approaches such as 16S rRNA genes and environmental genomics have illuminated black boxes in nutrient removal process and expanded our knowledge. However, most microbes responsible for the removal of phosphate and nitrogen such as *Accumulibacter* and *Nitrospira* remain uncultured. This is because optimum methodologies to concentrate these uncultured microbes and to obtain pure cultures have not been established. Here, we report a novel approach for physical enrichment of uncultured *Accumulibacter* and *Nitrospira* from microbial communities in activated sludge by a cell sorting system. Two scattering signatures representing forward scatter and side scatter of this system allowed morphological characterization of microbial particles in activated sludge. The distribution and size of microbial particles consisting of single cells, microcolonies, and aggregates depended on the levels of scattering signatures. Next generation sequencer and principal component analysis revealed each microbial population fractionated according to the levels of scattering signatures, resulting that uncultured *Accumulibacter* and *Nitrospira* could be sorted as single cells or microcolonies. Finally, quantitative fluorescence *in situ* hybridization analysis determined optimum fractions to collect sufficiently these target microbes from activated sludge. Consequently, this method would be very useful as an enrichment technique prior to isolation, genomic analysis, and physiological investigation of uncultured bacteria.

[**Key words:** *Accumulibacter*; Activated sludge; Aggregate; Cell sorter; Forward scatter; Microcolony; *Nitrospira*; Side scatter; Single cell]

The ecology and physiology of microbes in activated sludge of wastewater treatment plants (WWTPs) have been investigated to improve wastewater treatment ability. Removal of nitrogen and phosphate as nutrients in wastewater is an essential issue to prevent eutrophication (1). Accordingly, microbes responsible for the removal of nitrogen and phosphate have received a great deal of attention in the last few decades. Recently, development of molecular based techniques has revealed remarkable ecophysiology of uncultured polyphosphate-accumulating organisms (PAOs) and nitrifiers in activated sludge (2). Environmental genomics has also unveiled metabolic mechanisms of uncultured PAOs and nitrifiers (3–6). Although these molecular approaches definitely provide more beneficial information about unknown microbial ecology in activated sludge, abrupt deactivation of nitrogen and phosphate removal frequently occurs and hinders stable operation and maintenance in WWTPs. Therefore, obtaining enrichment and pure cultures of individual uncultured microbes is still required to unlock the biological nutrient removal process (7).

To date, many efforts to enrich and isolate uncultured PAOs and nitrifiers have been conducted. *Candidatus* Accumulibacter (hereafter *Accumulibacter*), which is recognized as one of the important PAOs, was highly enriched by controlling temperatures, pH and types of added organic compounds during enrichment process (8–11). As physical concentration techniques, density gradient centrifuge was effective for *Accumulibacter* (12). In nitrifiers, *Nitrospira* is the most dominant nitrite-oxidizing bacteria in activated sludge, but is slow-growing and recalcitrant in laboratories. Addition of low-concentration nitrite and ampicillin as antibiotics to a culture medium successfully repressed the growth of heterotrophic bacteria and *Nitrobacter* as the competitor of *Nitrospira*, resulting in high enrichment of *Nitrospira* (13). Subsequently, a micro-tweezers system allowed the separation of planktonic single cells of *Nitrospira* from the enrichment (14).

A cell sorter is also effective to separate specific microbial groups in the environment. This approach enabled enrichment of low concentration populations for subsequent experiments (15,16). Many types of fluorescence and scattering signatures have led to isolation of photosynthetic microbes based on photosynthetic pigments (17) and separation of target microbes by labeling extracellular substances such as proteins or glucose with fluorescence (18). However, these methods were not practical for microbes with no pigment or unknown microbes. Moreover, damage to cells with specific fluorescence prevented subsequent subculture or growth experiments. Therefore, a methodology to obtain target microbes without invasive manipulation of cells is required.

Previously, our research group successfully isolated uncultured *Nitrospira* from activated sludge using a combination of continuous feeding bioreactors and cell sorting techniques. Two phylogenetically distinct *Nitrospira* were selectively enriched by continuous

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feeding of low-concentration nitrite (19). Enrichment samples were then dispersed by weak sonication, resulting in categorization of three morphological types: (i) single-species microcolonies (*Nitrospira* cells); (ii) uneven shaped multi-species aggregates; and (iii) planktonic single cells. These morphological characteristics were exploited to sort this target bacteria from the microbial community. Briefly, *Nitrospira* microcolonies were detected and separated from microbial mixtures based on their distinct light scattering signature through the cell sorting system without specific fluorescent labeling. Forward scatter (FSC) and side scatter (SSC) enabled detection and sorting of *Nitrospira* single-species microcolonies (20,21). However, the cell sorter was applied after the culture-based enrichment process over several years, which would be laborious and difficult for many researchers. In next challenges, we investigated whether target bacteria could be enriched physically by exploiting morphological features of microbes present in sludge samples without the culture-based enrichment process.

Consequently, the current study was conducted to identify conditions for sorting single cells or microcolonies of *Accumulibacter* and *Nitrospira* from activated sludge samples via a cell sorting system. To our knowledge, this study is the first report in physical enrichment of uncultured *Accumulibacter* and *Nitrospira* from the same samples without cultivation and specific labeling. First, we applied activated sludge samples to a cell sorting system and fractionated them based on the magnitude of FSC and SSC. Microbial communities in each fraction were then analyzed using a next generation sequencer (NGS), which enabled phylogeny to be linked with morphological features. Subsequently, principal component analysis (PCA) based on the obtained sequence data revealed that uncultured *Accumulibacter* and *Nitrospira* could be sorted as single cells or microcolonies. The sorted *Accumulibacter* and *Nitrospira* cells were quantified by fluorescence *in situ* hybridization (FISH). Finally, fractions to collect sufficiently these target microbes from activated sludge were determined.

MATERIALS AND METHODS

Sample preparation Activated sludge samples were collected from a local municipal WWTP in Japan where an anaerobic/anoxic/oxic process was adopted. The samples were obtained on different date (May 30, June 9, and July 15, 2013) to confirm the reproducibility and were transported to our laboratory in opaque containers to prevent exposure to light. Upon arrival in the laboratory, 10 mL sludge samples were placed in 15 mL centrifuge tubes (Asahi Glass Co., Ltd., Tokyo, Japan) and dispersed by ultrasonic treatment (Sonifier II model 150; Branson, Danbury, CT, USA) for 3 min at an intensity of 7. Subsequently, the samples were filtered through a Nylon Net Filter (Cat No. NY2004700, 20 μm, Millipore) to remove relatively large flocs.

Cell sorting We used a FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA) cell sorter. Briefly, filtered samples were diluted with Milli-Q, then applied to the cell sorter at a sample flow rate adjusted to under 1000 events per second in single cell mode. A dot-plot was generated for FSC versus SSC by analyzing at enough events. We decided to divide evenly FSC and SSC values to investigate how microbial community and morphological condition changed according to both values. The both values were categorized with three levels consisting of low ($<5 \times 10^2$), middle (5×10^2–1×10^4), and high ($>1 \times 10^4$). The dot plot was then separated into nine areas (P1–P9), after which 20,000 events per area were sorted from seven areas (P1, P2, P4, P5, P6, P8, and P9), except for P3 and P7, for which there was no detection upon sequence analysis. Subsequently, 100 events per area were mounted onto glass slides for microscopic observation and FISH analysis.

Microscopic observation The sorted samples were placed onto glass slides and then dehydrated and stained with SYTOX Green nucleic acid stain (Life Technologies, Carlsbad, CA, USA). A fluorescence microscope (Axioskop 2 plus; Carl Zeiss, Oberkochen, Germany) was subsequently used to determine the ratio of single cells, microcolonies and aggregates to total events. Furthermore, a confocal laser scanning microscope (IX71; Olympus, Tokyo, Japan) was used to measure the cross-section area and height of each particle, after which the volume of each particle was calculated.

Polymerase chain reaction The sorted 20,000 events per area samples were placed in 1.5 mL tubes and centrifuged (15,000 rpm, 10 min). Next, the supernatants were discarded and 18 μL of 50 mM NaOH was added to tubes. These mixed samples were subsequently incubated at 95°C for 10 min, after which 2 μL of 1 M Tris–HCl (pH 8.0) was added. The V1, V2 hyper variable regions in the 16S rRNA gene of these samples were then amplified using the following primers, forward primer: 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNNNGA-TAGRGTTTGATCMTGGCTCAG-3′, reverse primer: 5′-CCTCTCTATGGGCAGTCGGT-GATCTGCTGCCTYCCGTA-3′. The forward primer contained Ion Torrent Primer A-key adaptor sequence, the broadly conserved bacterial primer 27F, a unique barcode sequence (NNNNNNNNNN) composed of 10 nucleotides, and a "GAT" inserted as a linker between the barcode and the rRNA primer. The reverse primer contained Ion Torrent Primer P1-key adaptor sequence, the bacterial primer 338R, and a "GAT" inserted as a linker between the barcode and the rRNA primer (Table S1) (22). The polymerase chain reaction (PCR) mixtures (20 μL) contained 10 μL of 2× PCR buffer for KOD FX Neo (Toyobo, Osaka, Japan), 4 μL of 2 mM dNTPs, 0. 25 μM of each primer, 5.25 μL of cell lysate, and 0.25 U of KOD FX Neo. A thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA) and the following thermal profiles were used for 16S rRNA gene amplification, which consisted of initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and elongation at 68°C for 1 min.

NGS analysis The amplified PCR products were purified using the Wizard SV Gel and PCR Clean-UP System (Promega, Tokyo, Japan). Size selection (insert sizes 400–450 bp) was performed using an E-Gel System with E-Gel SizeSelect 2% agarose gel (Life Technologies Ltd., Paisley, UK). The concentration of the purified libraries was measured using an Agilent 2100 Bioanalyzer with a High Sensitivity DNA chip (Agilent Technologies, UK Ltd., Stockport, UK). Individual libraries were diluted to 26 pM for template preparation using a OneTouch Template 400 kit (Life Technologies Ltd.).

According to the user guide (Catalog number: 4479878, Revision 4.0), emulsion PCR was conducted using the Ion PGM Template OT2 400 Kit (Life Technologies Ltd.). The libraries were then sequenced using an Ion PGM Sequencing 400 Kit (Life Technologies Ltd.) and the Ion Torrent PGM system according to the manufacture's protocols (catalog no. 4482002, revision 2.0) using 850 flows (generating an approximately 400–450 bp read length) on a 314 sequencing chip.

Subsequently, barcode sequences and forward primer of the obtained sequences were trimmed using CLC Genomics Workbench 8. In addition, sequences that did not have a quality score above 19 and a sequence length 300–400 bp were discarded. The library sequence data were converted into data format for Quantitative Insights Into Microbial Ecology (QIIME) (23). The QIIME pipeline takes in bar-coded sequence reads, separating them into individual communities by barcodes. Sequences with a similarity over 97% are considered the same operational taxonomic units (OTUs). Based on the obtained OTUs data, charts for statistical analysis were drawn using the 'R' software environment (version 3.0.1: http://www.r-project.org/). PCA was performed by R's prcomp function with standard parameters.

FISH analysis FISH analysis was conducted according to Amann's protocol (24). Briefly, sorted samples were placed on glass slides and dehydrated by soaking in 50%, 80%, and 98% ethanol for 3 min, respectively. Next, 100 μL of hybridization buffer (pH 7.2) composed of 35% formamide, 0.9 M NaCl, 0.01% SDS, and 20 mM Tris/HCl and 12.4 μL of oligonucleotide probes were mixed. The applied oligonucleotide probes were Ntspa 1431 specific for sublineage I of *Nitrospira*: 5′-TTGGCTTGGGCGACTTCA-3′ (25), a mixture of four probes specific for *Accumulibacter*, PAO462: 5′-CCGTCATCTACWCAGGGTATTAAC-3′, PAO651: 5′-CCCTCTGCCAAACTCCAG-3′, PAO846: 5′-GTTAGCTACGGCACTAAAAGG-3′ (26), and Acc623: 5′-CCAGCTGGACAGTCTCAA-3′ (27), or a mixture of two probes specific for most *Deltaproteobacteria* and most *Gemmatimonadetes* and the competitor, DELTA495a: 5′-AGTTAGCCGGTGCTTCCT-3′ and DELTA495a Competitor: 5′-AGTTAGCCGGTGCTTCTT-3′ (28,29). These oligonucleotides were fluorescently-labeled at the 5′ end by hydrophilic sulfoindocyanine dye (Cy3) (Fasmac Co. Ltd., Atsugi, Japan). Samples were hybridized in the above mixtures at 46°C for 2.5 h, then washed with washing buffer (0.112 M NaCl, 0.01% SDS, 20 mM Tris/HCl, pH 7.2) at 48°C for 16 min. Next, samples were washed with Milli-Q and stained with SYTOX Green nucleic acid stain. Stained cells and events of samples sorted by a cell sorter were counted using a fluorescence microscope (Axioskop 2 plus; Carl Zeiss).

RESULTS AND DISCUSSION

Cell sorting based on scattering signature Samples collected from the activated sludge tank of a WWTP were dispersed by weak sonication and filtered out to remove flocs and debris. The samples were then applied to a cell sorter at a flow rate of 200–300 events per second in single cell mode, after which the dot plot area was identified (Fig. 1A). Two scattering signatures representing FSC and SSC were used to sort microbial particles. In principle, the magnitude of FSC and SSC signals was correlated with the size of the particles and their complexity, respectively. The identified dot plot area was divided into nine areas (P1–P9) according to the magnitude of FSC and SSC (Fig. 1B), after which 100 events from each area were sorted and mounted on glass slides, stained with

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