JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 99, No. 4, 403–407. 2005
DOI: 10.1263/jbb.99.403

Characterization of Denitrifying Polyphosphate-Accumulating Organisms in Activated Sludge Based on Nitrite Reductase Gene

SATOSHI TSUNEDA,[1]* RYUKI MIYAUCHI,[1]
TAKASHI OHNO,[1] AND AKIRA HIRATA[1]

Department of Chemical Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan[1]

Received 13 September 2004/Accepted 24 January 2005

Nitrite reductase gene (*nirS*) fragments in the activated sludge obtained from a sequencing batch reactor (SBR) under anaerobic-aerobic condition were cloned and classified by restriction fragment length polymorphism (RFLP) analysis, and representative fragments were sequenced. One of the *nirS* clones was approximately 70% of all *nirS* clones in anaerobic/aerobic (existing oxygen and nitrate) cycle operation in which a large amount of anoxic phosphate uptake was observed. Although the activated sludge samples analyzed might contain bacteria that did not accumulate polyphosphate, it was likely that this *nirS* fragment sequence was that from denitrifying polyphosphate-accumulating organisms (DNPAOs) which can utilize both oxygen and nitrate as electron acceptors. The sequence was similar to the *nirS* sequences of *Thauera mechernichensis* (83% similarity) and *Azoarcus tolulyticus* (83% similarity) both of which belong to the *Rhodocyclus* group.

[**Key words:** activated sludge, enhanced biological phosphorus removal (EBPR), denitrifying polyphosphate-accumulating organisms (DNPAOs), polyphosphate-accumulating organisms (PAOs), nitrite reductase gene (*nirS*), polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP)]

Enhanced biological phosphorus removal (EBPR) processes for wastewater have been widely used over the past several decades because of their low cost compared with chemical treatment methods. EBPR is characterized by the presence of organisms referred to as polyphosphate-accumulating organisms (PAOs) that are capable of accumulating polyphosphate as an intracellular storage compound using oxygen as an electron acceptor. However, EBPR processes combined with biological nitrogen removal processes have a drawback in that organic substrate acts as a limiting factor for phosphorus release and denitrification at a low C/N ratio. Recently, the occurrence of denitrifying polyphosphate-accumulating organisms (DNPAOs) capable of utilizing nitrate instead of oxygen as an electron acceptor for phosphorus uptake has been reported (1–3). The use of DNPAOs in biological nutrient removal (BNR) processes is advantageous because the identical organic substrate can be efficiently used as energy source for both nitrogen and phosphorus removals. Other advantages associated with DNPAO activity include a reduction in surplus sludge production (4).

However, the DNPAOs as well as PAOs have not been isolated yet (or none of the isolated DNPAOs and PAOs could assimilate acetate and synthesize polyhydroxy-alcanoates [PHA] anaerobically concomitant with phosphorus release, which were different from EBPR sludge behavior [5]) and thus the biochemistry and genetics of PAOs have not been elucidated. Recently, it has been demonstrated using molecular techniques based on the rRNA phylogenetic framework that when efficient phosphorus removal is achieved in acetate-fed laboratory-scale reactors under cyclic anaerobic/aerobic or anaerobic/anoxic conditions, an organism closely related to *Rhodocyclus* (proposed name, *Candidatus* 'Accumulibacter phosphates' [6]) was abundant in these reactors (3, 7). Moreover, to investigate biomarkers for DNPAOs, monitoring of quinine profiles was done during enrichment of DNPAOs in biological phosphate removal systems (8). Although rRNA-targeted molecular analyses and fingerprinting of various biomarkers such as quinones reveal the population structure of activated sludge, they are unable to specify the organisms that have the ability to denitrify concomitant with phosphorus uptake.

The aim of this study is to obtain a better understanding of population dynamics of DNPAOs by identifying sequences of functional gene fragments specific for DNPAOs. The characteristic of DNPAOs that differentiates them from other PAOs is their ability to denitrify. Recently, PCR primer sets specific for functional genes involved in denitrification, namely, *nirS* and *nirK*, which encode cytochrome cd_1- and copper-containing nitrite reductases, respectively (9, 10)

* Corresponding author. e-mail: stsuneda@waseda.jp
phone: +81-(0)3-5286-3210 fax: +81-(0)3-3209-3680

and *nosZ*, which encodes nitrous oxide reductase (11), have been developed. The PCR method to detect *nir* genes was highly specific for *nirS* (12). To evaluate community changes of denitrifiers including DNPAOs, it is likely that specific primer sets for *nirS* are suitable. Therefore, in this research, diversity of *nirS* gene fragments was analyzed in DNPAO-rich sludge.

MATERIALS AND METHODS

Sequencing batch reactor operation A sequencing batch reactor (SBR) with a 2 *l* working volume was operated using the anaerobic/aerobic cycle at room temperature. Activated sludge withdrawn from an aerobic basin of a local domestic wastewater treatment plant (treating only COD) was inoculated prior to SBR operation. The reactor was operated with an 8 h cycle that consisted of a 15 min filling time, a 90 min anaerobic condition, a 285 min aerobic condition, a 65 min settling time and a 25 min withdrawing time. Since both influent and effluent volumes were 1 *l*, 16 h of hydraulic retention was maintained. Mixed liquor was removed at the end of the aerobic condition so that the 20 d of sludge retention was maintained. During the initial 3 d, 4 mg/*l* *n*-allylthiourea was added to the reactor at the start of the aerobic conditions to inhibit nitrification. During the subsequent days, nitrification occurred, resulting in the existence of both oxygen and nitrate as electron acceptors under the aerobic conditions. Synthetic wastewater of the following composition was used as the feeding solution: 300 mg of CH_3COONa, 65 mg of KH_2PO_4, 90 mg of $MgSO_4$, 140 mg of $(NH_4)_2SO_4$, 15 mg of $CaCl_2$, 1 mg of yeast extract, and 0.3 ml of nutrient solution (13) per liter.

Evaluation of phosphate uptake ability A batch experiment was conducted to evaluate aerobic and anoxic phosphate uptake abilities of the sludge in the SBR. The activated sludge (100 ml) sampled at the end of anaerobic conditions was divided and placed into two batch reactors, where nitrogen gas was purged for approximately 20 min to remove dissolved oxygen (DO) and it was confirmed that DO concentrations in the reactors were 0 mg/*l*. Then, one sample was exposed to aerobic conditions and the other to anoxic conditions (with the supplementation of 20 mg-N/*l* $NaNO_3$ in the reactor) for 285 min. The amounts of phosphate uptake per cell weight were estimated from the difference between the initial and final phosphate concentrations per mixed liquor suspended solid (MLSS) during an aerobic or an anoxic condition in the reactors.

Analytical methods The analyses of MLSS and phosphorus were performed according to standard methods (14). Nitrate and nitrite concentrations were determined with a high-performance liquid chromatograph equipped with an anion column (IC-Anion-PW; Tosoh, Tokyo) and an ultraviolet detector (230 nm) (UV-8011; Tosoh, Tokyo). Acetate was quantified as total organic carbon (TOC) using an automatic TOC analyzer (TOC-5000A; Shimadzu, Kyoto). These were measured on the days when batch experiments for determining phosphate uptake abilities were performed.

Acquisition of *nirS* gene fragments DNAs were extracted from activated sludge samples on days 2 and 28 (designated as samples A and B, respectively) using isoplant (Nippon Gene, Tokyo) following the manufacturer's instructions. The fragments of *nirS* genes (approximately 890 bp) were amplified using primers nirS1F and nirS6R (10). PCR amplification was conducted in an automated thermal cycler (icycler; Bio-Rad Laboratories, Hercules, CA, USA) using the following protocol: initial denaturation for 5 min at 94°C and 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 54°C, extension for 1 min at 72°C, followed by a final extension for 7 min at 72°C. A PCR mixture had a final volume of 50 µl, which contained 5 µl of 10×PCR buffer (containing 20 mM Mg^{2+}), 4 µl of dNTP mixture (2.5 mM each), 1.25 U of TaKaRa Ex Taq polymerase (Takara Bio, Otsu), 0.5 mM of each primer, and 219 ng of total DNAs extracted from sample A or 175 ng from sample B. PCR products were purified using SUPREC^{TM}02 (Takara Bio). Eluted PCR products were cloned using a Qiagen PCR cloning^{plus} kit (Qiagen, Valencia, CA, USA). A small amount of cell material picked up with a toothpick was resuspended in a prepared 20 µl PCR mix (Insert check-Ready-; Toyobo, Osaka). Inserts were amplified according to the manufacturer's instructions. Aliquots were electrophoresed on 1.5% (wt/vol) agarose gels. PCR products (0.005 µl) from clones containing inserts of the estimated size were further used for nested PCR amplification. In this step, the *nirS* primer pair and the same conditions were used as described for the initial PCR. Aliquots were electrophoresed on 1.5% (wt/vol) agarose gels.

RFLP analysis of *nirS* clones Restriction fragment length polymorphism (RFLP) analysis was conducted to screen PCR products. PCR products were digested in two reactions separately using restriction enzymes *Hha*I and *Msp*I at 37°C overnight. Digested products were electrophoresed on 3.5% (wt/vol) Metaphor agarose gels (Cambrex, East Rutherford, NJ, USA) in 1×Tris-borate-EDTA buffer for approximately 80 min at 50 V. After electrophoresis, gels were stained with SYBR Gold (Cambrex) for 30 min, and then visualized on an UV transilluminator. The RFLP patterns were compared visually and clones showing identical RFLP patterns were grouped into operational taxonomic units (OTUs).

Sequencing of *nirS* products and phylogenetic analysis Representatives of each OTU were selected for sequencing. DNA fragments were purified using a Wizard SV gel and a PCR clean-up system (Promega, Madison, WI, USA) and sequenced with primers nirS1F and nirS6R using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100-*Avant* genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers from AB185906 to AB185913. The sequences and some *nirS* sequence fragments obtained from DNA Data Bank of Japan (DDBJ) Database were aligned using the CLUSTAL W program (15), and a phylogenetic tree was constructed by the neighbor-joining method (16).

RESULTS AND DISCUSSION

Enrichment of DNPAOs Phosphate uptake was regularly measured in the presence of O_2 or nitrate as the electron acceptor. Figure 1 shows changes in phosphate uptake under the anoxic and aerobic conditions and phosphorus contents in the sludge (percentages of phosphorus weight in the sludge). At the start of operation, the amounts of phosphate taken up under both aerobic and anoxic conditions were as small as 2 mg-P/g-MLSS. During the first 10 d, during which nitrification was inhibited, the amount of phosphate taken up under the aerobic condition slightly increased but the amount of phosphate taken up under the anoxic condition was unchanged.

From day 10 to the end of the operation (day 36), nitrate concentrations at the end of aerobic conditions were always kept at 3–10 mg-N/*l* (data not shown), suggesting that nitrification occurred under the aerobic condition. Therefore, a small amount of nitrate existed at the start of anaerobic conditions, where DO concentration in the reactor was 0 mg/*l* (under detection limit), in the next cycle. Our previous study