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Stoichiometric and molecular evidence for the enrichment of anaerobic ammonium oxidizing bacteria from wastewater treatment plant sludge samples

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ABSTRACT

Anammox enrichments were readily developed from seven municipal wastewater treatment plants (WWTPs) sludge, but not with methanogenic granular sludge from two agro-industrial WWTPs. Only 50 d was required for the first evidence of anammox activity from a return activated sludge obtained from a WWTP operated for nutrient removal. The molar ratios of nitrite and ammonium consumption of approximately 1.32 as well as nitrate and dinitrogen gas product ratios of approximately 0.095 provided evidence of the anammox reaction. The presence of anammox was confirmed by polymerase chain reaction (PCR) using primer sets (PLA46F and AMX820R) specific for anammox bacteria. The 16S rRNA gene fragment of anammox bacteria was detected in seven enrichment cultures (ECs) with demonstrated anammox activity but not in the original inocula from which the ECs were derived and also not in the two methanogenic sludge samples, which indicates the PCR predicted the anammox activity. Two genera, *Brocadia* and *Kuenenia*, were successfully identified as the *Planctomycetes* occurring in the clone libraries of successful anammox enrichments. *Brocadia* dominated in cultures that were respiked extensively; whereas *Kuenenia* predominated in cultures that were less aggressively respiked. These findings indicate that respiking management may play an important role on selecting the genus of anammox bacteria. The batch enrichment results clearly illustrate that anammox can be readily enriched from municipal sludge from a wide variety of process operations at WWTPs.

1. Introduction

Ammonium is an important contaminant in a large variety of water systems (Henze and Comeau, 2008; Renou et al., 2008). The biological removal of NH_4^+ typically combines aerobic nitrification with anoxic denitrification at wastewater treatment plants (WWTPs) (Ekama and Wentzel, 2008). However, nitrification–denitrification has as a drawback the high input of energy for aeration required for the oxidation of NH_4^+ to nitrate, and the addition of readily biodegradable organic substrates to support denitrifying bacteria. The microbial process of anaerobic ammonium oxidation (anammox) provides a more sustainable and cost-effective alternative to improve traditional biological nutrient-nitrogen removal (BNNR) (Kartal et al., 2010). Anammox is catalyzed by chemolithoautotrophic bacteria (Eq. (1)) (Strous et al., 1998), involving the reaction of NH_4^+ with nitrite to produce N_2 under anaerobic conditions.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

The anammox process saves significant costs by requiring less aeration in order only about half of the NH_4^+ needs to be oxidized to NO_2^-. Costs are also potentially saved since no exogenous electron donor is required. Although anammox bacteria have a slow growth rate with reported doubling times around 10–12 d (Strous et al., 1998; van der Star et al., 2007), they can achieve a specific activity higher than 1 g N g^{-1} biomass dry weight d^{-1} (Strous et al., 1998). Therefore, high loading rates can be achieved for nutrient removal in anammox bioreactors, e.g. 26 kg N m^{-3} reactor d^{-1} (Tsushima et al., 2007).

Anammox bacteria belong to the phylum *Planctomycetes* (Jetten et al., 2005b). Anammox enrichments have been cultivated from various wastewater sources (Strous et al., 1997; Toh and Ashbolt, 2002; van der Star et al., 2007; Sanchez-Melsio et al., 2009). Often, the enrichment process is generally time-consuming lasting many months to years for success.

To shorten the start-up process, it is critical to identify suitable inocula, in which anammox bacteria are most likely present. Likewise cultivation strategies, which rapidly enable enrichment of anammox biomass, are also needed. The primary aim of this research was to rapidly enrich anammox from various types of

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WWTP sludge utilizing both stoichiometric and molecular evidence to confirm the developed anammox population. The second aim was to characterize the anammox microbial community and explore factors involved in the selection of anammox bacteria.

2. Materials and methods

2.1. Microorganisms

Nine different sludge samples obtained from different WWTPs were used as inocula in the batch bioassays. Aerobic activated sludge (AS-Ina), return activated sludge (RAS-Ina) and anaerobi cally digested sewage sludge (ADS-Ina) were obtained from a local municipal WWTP (Ina Road, Tucson, Arizona). The oxidation ditch sludge (ODS) was collected from another local municipal WWTP (Green Valley, Tucson, AZ). Three additional sludge samples were provided by Los Angeles County Sanitation District including two aerobic return activated sludge samples (RAS-SJ and RAS-Val) from municipal WWTPs in San Jose (San Jose, CA) and Valencia (Valencia, CA), respectively; and one sludge sample from a membrane biological reactor (MBR) from a municipal WWTP in Lancaster (Lancaster, CA). Methanogenic granular sludge (biofilms pellets) samples were obtained from industrial upward-flow anaerobic sludge blanket treatment plants treating alcohol distillery wastewater (NGS) (Nedalco, Bergen op Zoom, The Netherlands) and brewery wastewater (MGS) (Mahou beer brewery, Guadalajara, Spain). The volatile suspended solids (VSS) content of the sludge samples were 0.65%, 0.25%, 1.21%, 0.42%, 0.36%, 0.35%, 0.52%, 6.35% and 8.13% of the wet weight for AS-Ina, RAS-Ina, ADS-Ina, ODS, RAS-SJ, RAS-Val, MBR, NGS and MGS, respectively. An anam mox enrichment was obtained from a laboratory anammox se quence batch bioreactor from the Universidad de Santiago de Compostela, Spain. The inocula were stored at 4 °C.

2.2. Basal medium

The basal mineral medium was prepared using ultrapure water (Milli-Q system, Millipore) and contained the following compounds (mg L^{-1}): NH_4HCO_3 (213.6); $NaNO_2$ (246.4); $NaH_2PO_4{\cdot}H_2O$ (57.5); $CaCl_2{\cdot}2H_2O$ (100); $MgSO_4{\cdot}7H_2O$ (200); $NaHCO_3$ (2500); and 1.0 mL L^{-1} of two trace element solutions. Trace element solution 1 contains (in mg L^{-1}) $FeSO_4$ (5000) and ethylenediamine-tetraacetic acid (EDTA) (5000). Trace element solution 2 contains (in mg L^{-1}) EDTA (1500); $ZnSO_4{\cdot}7H_2O$ (430); $CoCl_2{\cdot}6H_2O$ (240); $MnCl_2$ (629); $CuSO_4{\cdot}5H_2O$ (250); $Na_2MoO_4{\cdot}2H_2O$ (220); $NiCl_2{\cdot}6H_20$ (190); $Na_2SeO_4{\cdot}10H_2O$ (210); H_3BO_3 (14); $NaWO_4{\cdot}2H_2O$ (50).

2.3. Screening bioassays for the enrichment of anammox cultures

Screening bioassays to develop anammox enrichment cultures (ECs) were incubated in shaken flasks at 100 rpm in a dark climate-controlled room at 30 ± 2 °C. Serum flasks (250 mL) were supplied with 200 mL basal mineral medium (pH 7.3) containing bicarbonate (2.5 g L^{-1}) as the only added C source as described above. The medium of full treatment was also supplemented with stoichiometric mixture as shown in Eq. (1) (1.32:1 mol NO_2^-: mol NH_4^+). NO_2^- and NH_4^+ were supplied at 3.57 mM and 2.71 mM, respectively. Inoculum was added to the assays to a final concentration of 1.5 g VSS L^{-1}. Various controls including abiotic controls (no inoculum), controls without NO_2^-, and controls without NH_4^+ were utilized in each experiment, details are provided in Supplemental material (SM). The flasks for the anaerobic assays were sealed with rubber stoppers, and then the medium and headspace were purged with N_2/CO_2 (80/20, v/v) for 20 min to exclude oxygen from the assay. All assays were conducted in duplicate. Liquid samples were analyzed for the concentration of NO_2^-, NO_3^- and NH_4^+.

2.4. Batch bioassays for anammox stoichiometry

Once the anammox ECs were successfully developed from the enrichment process, bioassays were set up to confirm anammox reaction by careful measurement of the stoichiometry. The set-up of the bioassay was the same as described for the screening experiment, except using serum flasks (160 mL) supplied with 100 mL medium and flushing headspace with He/CO_2 (80/20, v/v). Headspace samples were analyzed periodically for N_2 with a pressure lock gas tight syringe to confirm anammox reaction. Flushed headspace controls incubated with just water were monitored to ensure background levels of N_2 were low.

2.5. Polymerase chain reaction (PCR) amplification of the anammox bacteria

Community genomic DNA was extracted from 5 mL samples ta ken from each of the original sludge and the developed anammox ECs according to the method described by Sun et al. (2009). The success of DNA extraction was proven by amplification with uni versal bacterial primers of 27F and 1492R, which provided evi dence of the existence of bacterial DNA.

One specifically designed PCR primer set was used to target the 16S rRNA gene of anammox bacteria, including *Planctomycetes* specific forward primer PLA46F (5′-GGATTAGGCATGCAAGTC-3′) (Jet ten et al., 2005a) and *Brocadia* and *Kuenenia* specific reverse primer AMX-820R (5′-AAAACCCCTCTACTTAGTGCCC-3′) (Egli et al., 2001) . The PCR mixtures and running conditions are described in details in SM. The PCR products were purified using a Quick PCR purification kit (Qiagen, Valencia, CA), and then running gel electrophore sis on a 1.5% agarose gel containing 1× Tris-borate-EDTA buffer. The gels were stained with ethidium bromide (0.5 μg mL^{-1}) and were visualized under ultraviolet illumination in a MultiDoc-it imaging system (UVP, Upland, CA).

2.6. 16S rRNA gene clone library

The purified PCR products with primers of PLA46F and AMX -820R were cloned into plasmid vector pCR 2.1-TOPO using the TOPO TA cloning system (Invitrogen, Carlsbad, CA) to build clone library for five successful anammox ECs originating from RAS-Ina, RAS-SJ, RAS-Val, ODS and MBR, respectively. One more clone library was also built up for the anammox EC RAS-Ina with the amplified PCR products by universal bacteria primers of 27F and 1492R. The details of cloning and analysis were described in SM.

The number of clones analyzed for each culture was determined using a rarefaction curve to estimate the diversity (Analytic Rarefaction 1.3, UGA Stratigraphy Lab, University of Georgia, Atlanta, GA). An exponential model, $y = a\,[1 - \exp(-bx)]$, was used to fit the clone distribution data. The clones were clustered into phylo types on the basis of sequence similarity >99.5%. Sequence data were aligned with ClustalX, including 16S rRNA gene sequences from reference bacterial strains (GenBank) and unique phylotypes recovered from each of five established anammox enrichments, and a tree was constructed using PAUP* version 4.0b10. Selected clones amplified with primer set of PLA46F and AMX-820R representing five anammox bacterial phylotypes obtained in each culture have been deposited in the GenBank database. The GenBank accession numbers for the sequences used to prepare phylogentic trees are shown in SM.