Detection of *Planctomycete* Bacteria Capable of Autotrophic Nitrogen-removal in Activated Sludge and Biofilm

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Abstract: Three gene libraries of sludge samples from a municipal wastewater treatment plant and two lab-scale reactors for autotrophic Nitrogen-removal were constructed, targeting the partial *Planctomycete*-specific 16S rRNA. The results showed, two classes of *Planctomycete* bactaria are present in all of the samples, and in the autotrophic N-removal reactors, the class capable of catalyzing autotrophic N-removal process has been enriched.

Keywords: *Planctomycete*; Nitrogen-removal; 16S rRNA; wastewater treatment

1 Introduction

Completely autotrophic Nitrogen-removal under oxygen-limited conditions, is a promising novel option for low-cost ammonium removal from ammonia-rich wastewater [1-5].

Some early studies revealed that certain bacteria, belonging to the order *Planctomycetales*, such as Candidatus *Brocadia anammoxidans*[6] and Candidatus *Kuenenia Stuttgartiensis* [7], are the key population capable of simultaneous ammonia and nitrite removal under anoxic conditions. Some recent papers[8,9] also discovered that several strains of *Planctomycetales* were involved in the process.

During our past investigation, a completely autotrophic process was fulfilled through a suspended sludge reactor and a moving-bed biofilm reactor, and the full-cycle 16S rDNA approach was performed through using a universal primer set. By BLAST of the sequences retrieved from the gene library, the dominant clones F-OTU1 sequence (Accession No: AY369141) was affiliated with *Planctomycete* bacteria, the members of the order *Planctomycetales*[10]. Therefore, it was supposed that the F-OUT1 bacteria be the chief population responsible for the nitrite reduction during the autotrophic N-removal process.

In this paper, a Planctomycete-specific forward primer and a new-designed primer set based on the sequence of F-OTU1 were used for PCR with three sludge samples as templates. Based on the PCR products of the new primers, three gene libraries were constructed. We attempt to reveal the distribution of the Planctomycete bacteria and estimate the function of the mysterious organisms F-OTU1 during the N-removal process.

2 Materials and Methods

Three sludge samples in this study were collected: Sample C, from the aeration tank of a municipal wastewater treatment plant (Minhang, Shanghai, China); Sample S, from the suspended sludge reactor for autotrophic N-removal in our laboratory; Sample M, from the moving-bed biofilm reactor for autotrophic N-removal in our laboratory.

The reactors of sample S and sample M were both 12 L of volume. For the moving-bed reactor, a kind of spongy filling of approximately 1 cm^3 was applied. The influents (synthetic wastewater) of both reactors comprised a simple autotrophic medium, free of organic carbon, consisting of 21.4 mmol/L NH_4HCO_3 (equivalent to 300 mg NH_4^--N/L), 0.025 g/L KH_2PO_4, 0.3 g/L $CaCl_2$, 0.2 g/L $MgSO_4$, and 1 mL/L trace element solution [0.2 mmol/L $MnSO_4$, 0.8 mmol/L H_3BO_3, 0.15 mmol/L $ZnSO_4$, 0.03 mmol/L Na_2MoO_4, 2.5 mmol/L $FeSO_4$, 0.1 mmol/L $CuSO_4$, 1 mmol/L $Ni(NO_3)_2$, 0.24 g/L $CoCl_2$, 0.05 g/L $NaWO_4$]. By means of an automatic feedback controller, air was pumped from the bottom of the reactor to maintain the dissolved oxygen concentration at 0.5–1 mg/L, and the pH of the sludge suspension was kept at the range of 8.0–8.5. The total nitrogen removal were both up to 70 % when the sample S and M were collected.

Total genomic DNA was extracted following the protocol described by U. Purkhold *et al.* [11]. To increase the sensitivity, a nested PCR program was applied as described previously [12]. For first round PCR amplification of 16S rRNA genes of members of the *Planctomycete*, the set of recently published phylum-specific primer PLA46 in combination with the universal reverse primer 1390R was used [7]. The PCR consisted of 30 cycles (3 min at 94℃, 1 min at 52℃, and 2 min at 72℃) and one final cycle at 72℃ for 3 min. During the second round of PCR, the obtained fragments were reamplified by using primers Pla-F986 (5'-GAC TTG ACA TGC ATG GAT TAA-3') and Pla-R1392 (5'-GGC TCA GGA ACA

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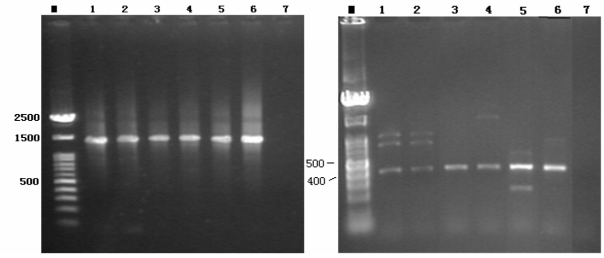
TAT TCA CCG C-3'), targeting the members of *Planctomycete* in autotrophic N-removal reactor, which were designed based on the sequence (AY369141) recovered from our previous study. The PCR consisted of 35 cycles (3 min at 94℃, 30 s at 57℃, and 45 s at 72℃) and one final cycle at 72℃ for 3 min.

The PCR products of the second round amplification were purified and then ligated into vector pUCm-T (T-A cloning kits, Biocolor, Shanghai), and the ligations were transformed into *E.coli* DH5α competent cells. Ten clones of every sludge sample were selected randomly and sequenced. The sequence data were compared with sequences in the GeneBank. Treeing and phylogenetic analysis were done using Clustal X [13] and Treeview [14].

3 Results and Discussion

The result of two rounds of PCR was showed in Fig.1. The products (approximately 1344bp) were observed as expected through the amplification of all three samples with PLA46 and 1390R, which was consistent with the previous work [5]. Furthermore, the target products of about 406 bp appeared distinctly through the second-round amplification.



(A) (B)

Figure 1. Direct and nested PCR amplifications of partial-16S rRNA gene fragment of *Planctomycete* from three sludge samples. Direct PCR amplification was performed with primers PLA46 and 1390R (A) and followed by nested PCR amplification with primers Pla-F986 and Pla-R1392 (B). Lane M: 3S 100bp DNA ladder; lane 1, 2: sample C; lane 3, 4: sample S; lane 5, 6: sample M; lane 7: negative control.

This result showed that the F-OTU1-like bacteria inhabit both the autotrophic N-removal reactor and the municipal wastewater treatment plant, which suggested that these bacteria are probably widespread.

The sequences determined in this study are available in GeneBank under accession No. AY598019—AY598027.

The results of BLAST comparisons showed all the 30 clones could be grouped roughly into two classes. The first could be affiliated with the clone F-OTU1, with which the sequence similarities were higher than 99%. This class included 17 clones, 1 of sample C, 8 of sample S, and 9 of sample M. The other class was similar with uncultured *Planctomycetales* sludge bacterium A17 (similarity >99%) in a nitrifying-denitrifying sludge and comprised 12 clones, among of which, 8 of sample C, 2 of sample S, and 1 of sample M. Besides, one clone of sample C showed novel phylogenetic character (similarity <90%). Approximately 406 nucleotides arranged from the inserts were launched into the successive phylogenetic analysis. Fig.2 shows the phylogenetic tree reflecting the relationships of microorganisms registered to the database.

The result of sequence analysis suggests that, after approximately one year of incubation and operation, the F-OTU1 bacteria of *Planctomycete*, which are capable of catalyzing the autotrophic N-removal process, were enriched in both the suspended sludge reactor and the moving-bed biofilm.

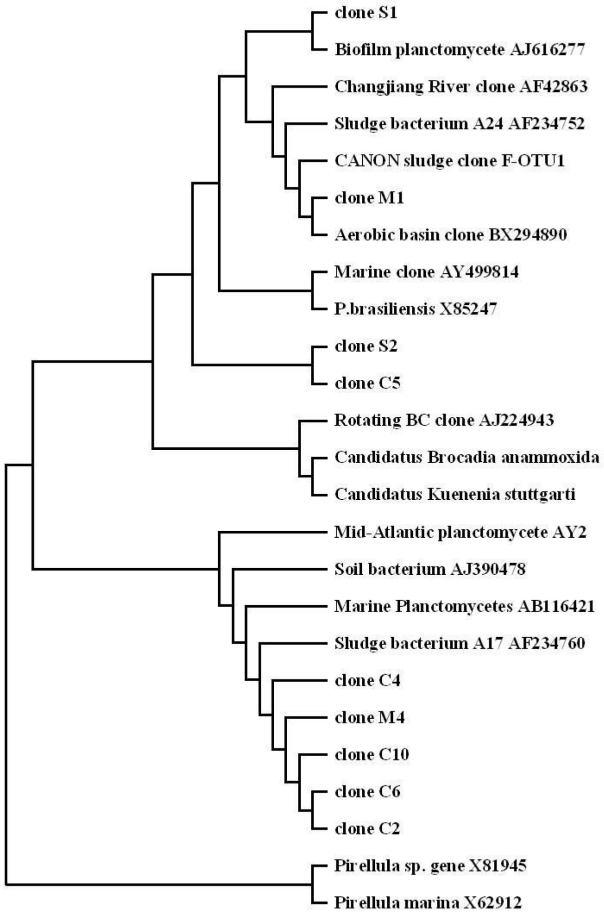


Figure 2. Phylogenetic tree for the clones derived from 3 samples. The tree was constructed from neighbor-joining analysis based on Planctomycete partial-16S rDNA sequences, depicting the relationships of the organisms in three sludge samples with their closest relatives.

In this paper, we attempted to use the new-designed primers to amplify specifically the F-OTU1 bacteria, however, the bacteria were detected in all three samples of sludge, and two lineages of *Planctomycete* were found in the gene library of the PCR products. It is suggested that the F-OTU1 bacteria, as a strain which takes the role of nitrogen-removal at the anoxic condition, exist in wide-spread water environment with nitrogen but not limit themselves within the autotrophic N-removal reactor. The reasons for their dominance in the autotrophic N-removal reactor is likely either these types of reactor and nutrition condition enrich the organism capable of catalyzing the autotrophic N-removal process, or a latent metabolic pathway concerning nitrogen- cycling was activated. As an evidence for that, the anammox bacterium Kuenenia stuttgartiensis are characterized by a compartmentalized cell architecture featuring a central cell compartment, the "anammoxo- some". Thus far unique "ladderane" lipid molecules have been identified as part of their membrane systems surrounding the different cellular compartments[15]. Nitrogen formation seems to involve the intermediary formation of hydrazine, a very reactive and toxic compound.

The family of *Planctomycete*s is a worthy-study microorganism for nitrogen-cycling and wastewater N-removal. So far as, they are found to be present and enriched in diverse scale wastewater treatment systems. The investigation for them should be performed in two aspects: first, unveil their intrinsic metabolic mechanism underlying the completely autotrophic N-removal, in especial, the switch of the metabolic pathway under the high-nitrogen-concentration condition; second, escalate the dissolved-oxygen threshold endured by the bacteria so that to make the systems more stable and robust.

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