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Full Length Research Paper

Molecular diversity analysis of planctomycete-like bacteria in inosine fermentation and municipal wastewater treatment systems

Hao Zhang, Ying Xu, Yifeng Ding, Qingxiang Yang*, Siwei Ren, Xueling Li, Xuanyu Chen, Yuanyuan Xu and Hongxin Hao

College of Life Sciences, Henan Normal University, Xinxiang 453007, China.

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In this study, the diversity of planctomycete-like bacteria in an inosine fermentation and a municipal wastewater treatment plants was investigated by denaturing gradient gel electrophoresis (DGGE) of nested polymerase chain reaction (nested PCR) amplified 16S rRNA gene fragments using planctomycete-specific forward primer and a bacteria universal reverse primer. The bacterial community structures in the two wastewater treatment systems were obviously different due to the different treatment processes and different wastewater characteristics. The highest similarity of the microbial community structure happened in the two samples from the acidification and the anaerobic tank in inosine fermentation plant reaching 67%. While the two samples from the anaerobic and the aerobic tank of municipal wastewater treatment plant reached 66%. Ten sequences were recovered from the DGGE gel and were assigned to Clostridia, Proteobacteria, Bacteroidete and Candidate division based on the phylogenetic analysis. The two sequences grouped into Candidate division had more than 92% similarities with the Planctomycete-like bacterial clones. However, no anaerobic ammonium oxidation (anammox) activities were detected in the samples of these two wastewater treatment plants.

Key words: Planctomycete-like bacteria, wastewater treatment, denaturing gradient gel electrophoresis (DGGE).

INTRODUCTION

Planctomycetales is a separate division in domain bacteria with special characteristics of cell structure, genetics and physiology, etc. Researches on the molecular ecology indicated that this group of bacteria had a wide distribution in nature, such as ocean settlings, fresh water ecosystem, wastewater treatment reactors and soil (Jetten et al., 2003; Fuerst, 2004; Fuerst and Sagulenko, 2011). All of them played important roles in the cycle of inorganic or organic substances (Jenkins et al., 2002). Up to now, only four cultured genera of Planctomycetales including *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera* have been described clearly and they are all aerobic chemoheterotrophs (Ward et al., 2006). However, in recent years, the physiological groups of Planctomycetales have been expanded as the research being deepened. Members of them include not only chemoorganotrophs and obligate or facultative aerobes but also obligate anaerobes and autotrophs (Kulichevskaya et al., 2007; Shu and Jiao, 2008). Especially in 1995, the anaerobic ammonium oxidation

*Corresponding author. E-mail: yangqx66@163.com. Tel/Fax: +86-373-332-5528.

Abbreviations: **DGGE**, Denaturing gradient gel electrophoresis; **PCR,** polymerase chain reaction; **Anammox**, anaerobic ammonium oxidation; **plant IF,** inosine fermentation wastewater treatment plant; **plant M,** municipal wastewater treatment plant; **ABR,** anaerobic baffled reactor; **UBF,** upflow blanket filter; **CASS,** cyclic activated sludge system; **HRT,** hydraulic retention time; **A/O,** anoxic-aerobic; **COD,** chemical oxygen demand; **TN,** total nitrogen; **TP,** total phosphate; **SS,** suspended solid substances.

(anammox) process was first described in a denitrifying pilot bioreactor in the wastewater treatment plant of Gist-Brocades (Mulder et al., 1995). At present, three uncultured genera of anammox bacteria belonging to a deep branching cluster within the Planctomycetales, *Candidatus Brocadia*, *Candidatus Kuenenia* and *Candidatus Scalindua* (Kuypers et al., 2003; Kuenen and Jetten, 2001; Schmid et al., 2003), have been paid close attentions by investigators due to their high efficiencies of removing nitrogen from nitrite and ammonium without requiring either organic matter or O_2 in wastewater treatment processes (Alexandre et al., 2009). But our knowledge on Planctomycetales is still limited today. So, investigation of Planctomycete-like bacteria in different environments should have great significance due to their abundant metabolic diversity. However, few researches about it were involved in industrial wastewater treatment systems, except Chouari (Chouari et al., 2003) and Innerebner (Innerebner et al., 2007).

Simultaneously, some molecular biological techniques such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), fluorescence in situ hybridization (FISH) and cloning of 16S rDNA have been developed and widely applied to analyze the genetic diversity of microbial communities in wastewater treatment systems giving much more information of microorganisms than traditional cultivation methods. DGGE resolves the differences of PCR-amplified regions of genes based on differences in nucleotide sequences (Lyautey et al., 2005) and the band patterns from DGGE map can directly reflect the genetic diversity of the microbes in certain samples (Sanz and Köchling, 2007). So, it has been widely used in molecular microbial ecology researches. To increase the sensitivity of such ecological analysis, the technique of nested PCR was developed to assess the diversity of the microbial species presence in low concentrations in environments (Liu et al., 2007).

In this study, a municipal and a typical inosine fermentation wastewater treatment plant were selected as cases to investigate the distribution and diversity characteristics of planctomycete-like bacteria in biological treatment systems using nested PCR-DGGE technique.

MATERIALS AND METHODS

System characterization

An inosine fermentation wastewater treatment plant (plant IF) and a municipal wastewater treatment plant (plant M) which locate in Xinxiang, Henan Province, China and have run stably for more than two years were selected as cases in this study. Plant IF produces 700 tons of high-strength and 1,300 tons of low-strength of effluents every day. Its wastewater treatment system is composed of an ABR (Anaerobic Baffled Reactor), 4 UBF (Upflow Blanket Filter) reactors and a CASS (Cyclic Activated Sludge System) tank treating 2000 m^3 wastewater per day as indicated in Figure 1. The high strength of the original wastewater was first fed into an acidification basin and then was distributed into the 4 UBF reactors with hydraulic retention time (HRT) of 53 h. The CASS tank receives the treated effluents from the UBF reactors and the original low strength of wastewater. Plant M is a typical A/O (anoxic-aerobic) process treating 150, 000 m^3 municipal wastewater per day.

Analytical methods

Water and activated sludge samples were collected from different units in the two wastewater treatment systems, immediately transported to the lab and stored at -20°C before the chemical and biological analysis (< 48 h). COD (chemical oxygen demand), NH_4^+-N, total nitrogen (TN), total phosphate (TP) and suspended solid substances (SS) were detected as the standard methods (APHA, 1995). The pH was determined using a digital pH meter. For each plant, the mainly physical and chemical characteristics of wastewater at every biological treatment stage are shown in Table 1.

DNA extraction

The activated sludge samples firstly needed to be washed three times using phosphate-buffered saline (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0) and then centrifuged at 4°C, 10,000 rpm for 15 min. The genomic DNA extraction was conducted using the phenol–chloroform method (Bourrain et al., 1999) and then checked by electrophoresis on a 0.8% (w/v) agarose gel. Nucleic acids were stored at -20°C.

Nested polymerase chain reaction (PCR) amplification

For planctomycete-like bacteria population analysis of activated sludge samples, a nested PCR technique was used to amplify 16S rRNA gene to increase the sensitivity. The first round of PCR was conducted using a Planctomycetales-specific forward primer PLA-46F (5'-GGATTAGGCATGCAAGTC-3') and a bacteria universal reverse primer 1378R (5'-GGGCGGWGTGTACAAGGC-3') as described by Pynaert et al. (2003) and Chouari et al. (2003). The second round of PCR was conducted using the above PCR products as templates and the primers GC-341F (5'-CGCCC GCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACG GGAGGCAGCAG-3') and 518R (5'-ATTACCG CGGCTGCTGG-3') to amplify the V3 region of 16S rDNA as the method of Cunliffe (Cunliffe and Kertesz, 2006).

Denaturing gradient gel electrophoresis (DGGE) analysis

The second PCR products were analyzed by DGGE using a Bio-Rad D-Code universal mutation system (Bio-Rad Laboratories, USA). DGGE was performed in 10% (w/v) polyacrylamide gels that contained a denaturing gradient of 30 to 60% denaturants. Electrophoresis was conducted at 25 V for 25 min, and then changed to a constant voltage of 75 V for 16 h. After electrophoresis, the gel was stained for 15 min in EB solution, then immediately observed and photographed by the Gel Documentation System.

The major bands on the DGGE gels were excised and cloned into the pMD 18-T vector for sequencing. The recombinant plasmid DNA was then extracted using standard procedures and sequenced by Sangon Biotech Company, Limited, Shanghai, CHINA. Analysis of the bacterial community on the DGGE fingerprints was conducted using the Quantity-One software package (Bio-Rad Laboratories, USA) according to the manufacturer's instructions.

Table 1. Main characteristics of wastewater in every treatment stage of plant IF and M.

Treatment system	pH[a]	COD[a] (mg/L)	NH_4^+-N[a] (mg/L)	TN[a] (mg/L)	TP[a] (mg/l)	SS[a] (mg/L)
Plant IF						
High-strength wastewater	4.7 - 6.5	3765.0 - 8130.0	111.7 - 171.1	242.7 - 336.5	31.8 - 66.5	2406.0- 3220.0
Adjusting tank	5.5 - 6.7	4455.0 - 8322.0	119.5 - 217.9	251.0 - 399.0	42.0 - 90.8	3810.0- 3909.0
Hydrolysis acidification tank	7.1 - 7.5	1530.0 - 5172.0	178.6- 213.5	184.9 - 430.3	22.9 - 46.3	6802.0- 9098.0
UBF	7.2 - 7.9	2592.0 - 5302.50	20.1- 54.4	209.6 - 351.1	26.7 - 46.9	14376.0- 27990.0
Low-strength wastewater	6.8 - 7.7	201.0 - 666.0	44.9- 86.1	21.7 - 83.2	0.2 - 0.5	853.0 - 1130.0
CASS	7.4 - 7.6	85.0- 231.0	32.2- 58.2	23.6 - 62.9	0.2 - 0.5	1989.0- 2760.0
Effluent	7.4 - 7.8	35.0- 156.0	18.0- 47.3	24.4 - 45.3	0.2 - 0.4	175.0- 284.0
Plant M						
Influent	7.2 - 7.7	133.3 - 281.0	33.4- 64.2	38.2 - 68.2	2.8 - 4.0	230.0- 405.0
Anoxic tank	7.0 - 7.2	45.0 - 115.7	22.8- 44.4	27.0 - 53.3	14.5 - 18.9	5104.0- 7098.0
Aerobic tank	6.8 - 7.2	18.5- 164.2	13.4- 24.9	24.8 - 35.2	1.4 - 2.5	5079.0- 8344.0
Effluent	7.0 - 7.4	9.8- 18.5	22.5- 27.1	26.4 - 36.6	0.2 - 0.7	15.0- 90.0

[a] maximum-minimum are shown.

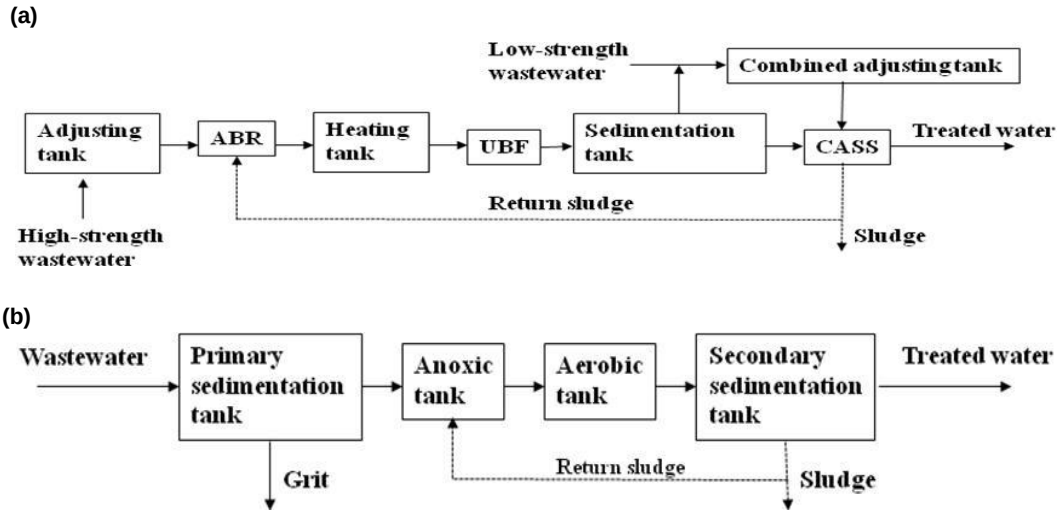

Figure 1. Flow charts of the inosine fermentation wastewater treatment system (a) and the municipal wastewater treatment system (b).

Phylogenetic analysis

All the nucleotide sequences were analyzed by conducting a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of the 16S rRNA genes of the isolates and their closest relatives were conducted using the Clustal X1.8 program. Phylogenetic trees were constructed by neighbor-joining (NJ) method with the Jukes-Cantor correction in MEGA 4.1 package.

Anaerobic batch experiments

Series of anaerobic batch experiments were carried out to detect the anaerobic ammonium oxidation (anammox) activities of the biomass samples from two wastewater treatment systems in 300 ml serum bottles containing 250 ml of mixed liquor as described by Pynaert et al. (2003).

RESULTS AND DISCUSSION

Analysis of the microbial community structures in the two wastewater treatment plants

Total DNA was successful extracted from the activated sludge samples originating from different biological treatment units in the two wastewater treatment systems of plant IF and M. The PCR-DGGE technique was used

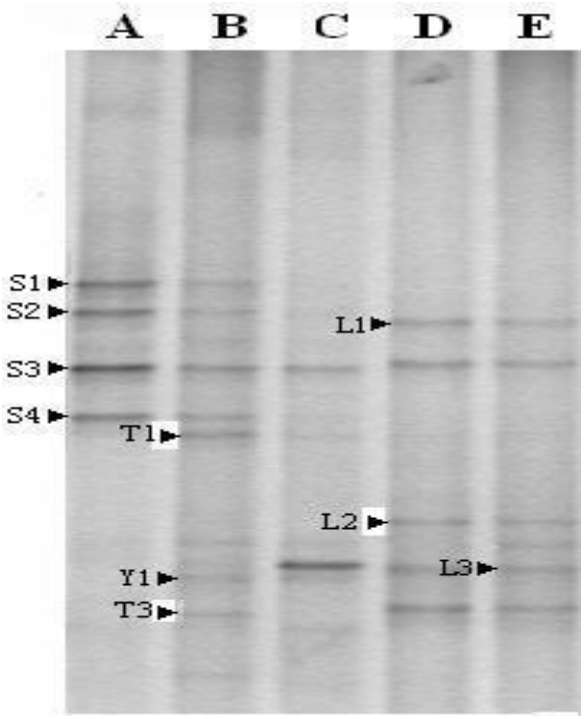

Figure 2. DGGE patterns of nested PCR products using the Planctomycete specific forward primer PLA-46F and the bacterial universal reverse primer 1378R. Samples from the acidification basin(A), anaerobic basin(B), aerobic basin (C) of plant IF and the anaerobic basin(D), aerobic basin (E) of plant M. Special bands were signed by black letters and arrows.

in this study to assess the bacterial community structures as described in materials and methods. The results are shown in Figure 2. On the gel used to conduct DGGE, various lanes contained different microbial samples collected from the treatment units. Bands in different positions in the electrophoresis gel were considered to represent unique species of microorganisms. Based on the DGGE profile, the diversities of the bacteria in different wastewater treatment samples were significantly different, but they were far lower than that in other reports on wastewater treatment systems (Alexandre et al., 2009; Liu et al., 2007) due to the planctomycete specific primer being used in the first round of PCR. Specifically, the highest diversity of the planctomycete-like bacteria occurred in the sample of the anaerobic unit in plant IF with at least 10 bands visible and the lowest one happened in the aerobic unit of this wastewater treatment system with only 5 bands distinguished by naked eyes, which suggested that an anaerobic condition might be helpful for the survival of this group of bacteria. However, the diversity of the two samples from the anaerobic and aerobic units in plant M had no significant difference, which was probably due to the shorter HRT and the returned sludge in the anaerobic tank.

Similarity analysis on the DGGE profile (Figure 3) indicated that the five samples formed two main clusters, in which the samples from the acidification and anaerobic tanks of plant IF were grouped together sharing the highest identity of 67%, the aerobic (CASS tank) sample in plant IF clustered together with the two samples in plant M sharing the identity of only 11%. Moreover, the aerobic sample in plant IF formed a separate branch in the second cluster with 31% identity with the other two samples. It is reasonable for the high identity between the acidification and anaerobic samples of plant IF that these two environments possessed similar anoxic and nutrient conditions and suitable for planctomycete-like bacteria survival.

However, the activated sludge samples from anoxic tank (Lane D) and aerobic tank (Lane E) in plant M also shared as high as 66% identity, which could be explained as the relatively shorter HRT in the two tanks and the frequent biomass exchange due to the returned sludge from the settling tank to the anaerobic tank. In the aerobic CASS tank in plant IF, the high concentration of dissolved oxygen and large amount of low strength of wastewater fed in, which formed a special ecological environment for microorganisms and resulted in lower diversity and identity of planctomycete-like bacteria compared with other basins.

Finally, the activated sludge in plant IF came from plant M at the beginning of the wastewater treatment system being set up, but after two years of adaptation and domestication by the fermentation effluents, the bacterial community structures have largely changed sharing no more than 31% similarity with the original sludge source.

Taken together, the distribution and diversity of planctomycete-like bacteria in different wastewater treatment system were significantly related to the sewage constituents and the operating factors such as HRT, dissolved oxygen concentration and flow or sludge returning.

Phylogenetic analysis

Here we only focused on the bright bands in the DGGE profile as indicated in the picture to obtain the information of the dominant planctomycete-like bacterial population in the two wastewater treatment systems. Specifically, total 10 bands were excised from the gel and sequenced for further analysis. Their sequences have been deposited in the GenBank database under the accession numbers of FJ950727, FJ950729 to FJ950736 and FJ968106. The 10 sequences were conducted a phylogenetic analysis using software Mega 4 and the results are shown in Figure 4.

Based on the phylogenetic tree, the 10 sequences were clustered into 6 different groups including Alphaproteobacteria, Betaproteobacteria, Gammaproteo-bacteria,Bacteroidetes, Clostridia and Candidate decision.

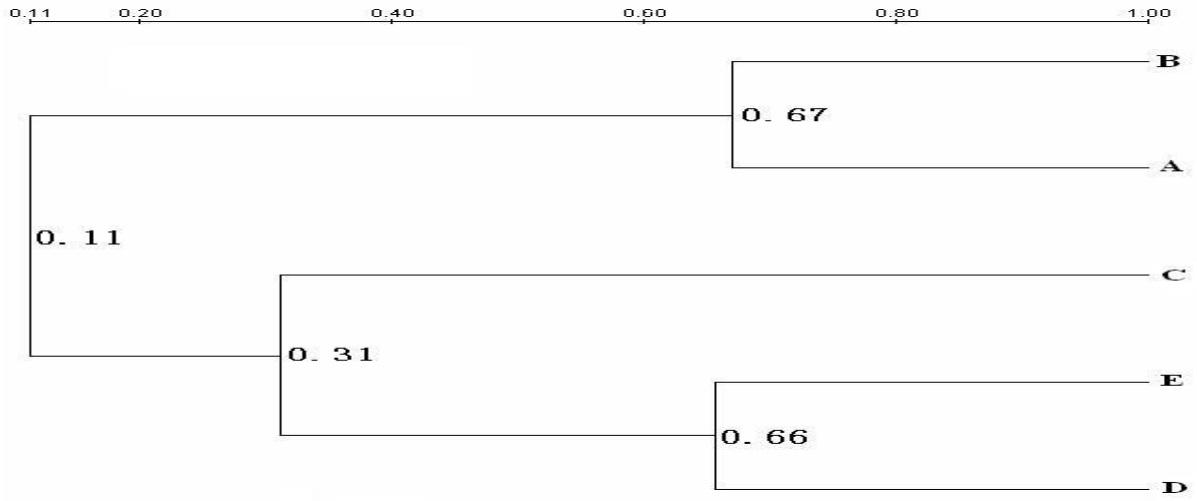

Figure 3. Cluster analysis of the DGGE profile using the complete linkage method.

Sequence S4 was assigned to the cluster of Alphaproteobacteria. The represented clone was present in the anaerobic basin in plant IF and shared 99% identity with an unpublished strain, *Rhodobacter changlensis* which was isolated from oil contaminated soil.

Clone L1 and T3 were only present and dominant in the aerobic and anaerobic basins of plant M. The represented sequence of L1 was clustered in the group of Betaproteobacteria and had 99% identity with an uncultured *Dechloromonas* sp. that was also retrieved from a municipal activated sludge sample (Yan et al., 2009). It was suggested that *Dechloromonas* sp. was probably a general bacterial species in the municipal wastewater treatment systems. The represented sequence of T3 was clustered in the group of Gammaproteobacteria and had 100% identity with a gammaproteobacterium clone retrieved from waste of a steel plant (Freitas et al., 2008).

Two sequences of S2 and Y1 were grouped in Bacteroidetes sharing 99 and 100% identities with the two different uncultured clones from a mesophilic anaerobic digester which treated municipal wastewater sludge (Riviere et al., 2009). These two clones presented only in plant IF and mainly in acidification and anaerobic basins in this study suggesting their predominance in anaerobic conditions.

Sequence L2, L3 and T1 were clustered into the group of Clostridia sharing 97 to 99% identities with some uncultured clones.

Two sequences, S1 and S3, were clustered into Candidate division and shared the maximum similarity of 98 and 100%, respectively, with an uncultured clone from a landfill leachate-polluted aquifer (Roling et al., 2001). Candidate division is an unclassified group of bacteria in which large amount of possible novel microorganisms are contained and very few pure strains are found till now. Further analysis showed that S1 and S3 exhibited more than 92% similarity with the two planctomycete-like bacterial clones (DQ393189 and DQ393190) obtained from the anaerobic basin of an alcohol plant wastewater treatment system in our previous report, in which the sludge samples showed low anammox activities even under the disadvantage environments for anammox microorganisms. However, the sludge samples in this study had no such activities based on our near one month continuous detection. Different from clone S1 only existing in typical anaerobic condition (acidification basin and anaerobic basin of plant IF), clone S3 was present in all the samples including aerobic and anaerobic conditions of the two wastewater treatment systems. It suggested that the represented strain of S1 was a strict anaerobe, while that of S3 was a facultative species.

Taken together, although the brightness of the bands is high enough in the DGGE gel, no sequence had enough scores to be assigned to the group of Planctomycete-like bacteria based on the blasting results from the website of http://www.ncbi.nlm.nih.gov/BLAST/. While extensive diversities of bacterial species were found in other order, which was probably resulted from the low specificity of PCR amplification by using Planctomycete-specific forward primer and a bacteria universal reverse primer. However, we tried the amplification using the planctomycete specific reverse primer instead of the universal one and no PCR band could be obtained. In our previous amplification of sludge samples from an alcohol fermentation wastewater treatment plant using the same set of primers, although lots of bacterial species in other orders were recovered still 16% planctomycete-like species were obtained. The reasons for this phenomenon may be in two sides. Specifically, there was indeed no planctomycete-like bacterium in the inosine fermentation wastewater treatment system, or some species were too weak to be detected by DGGE method while they might be detected by picking and sequencing enough quantities

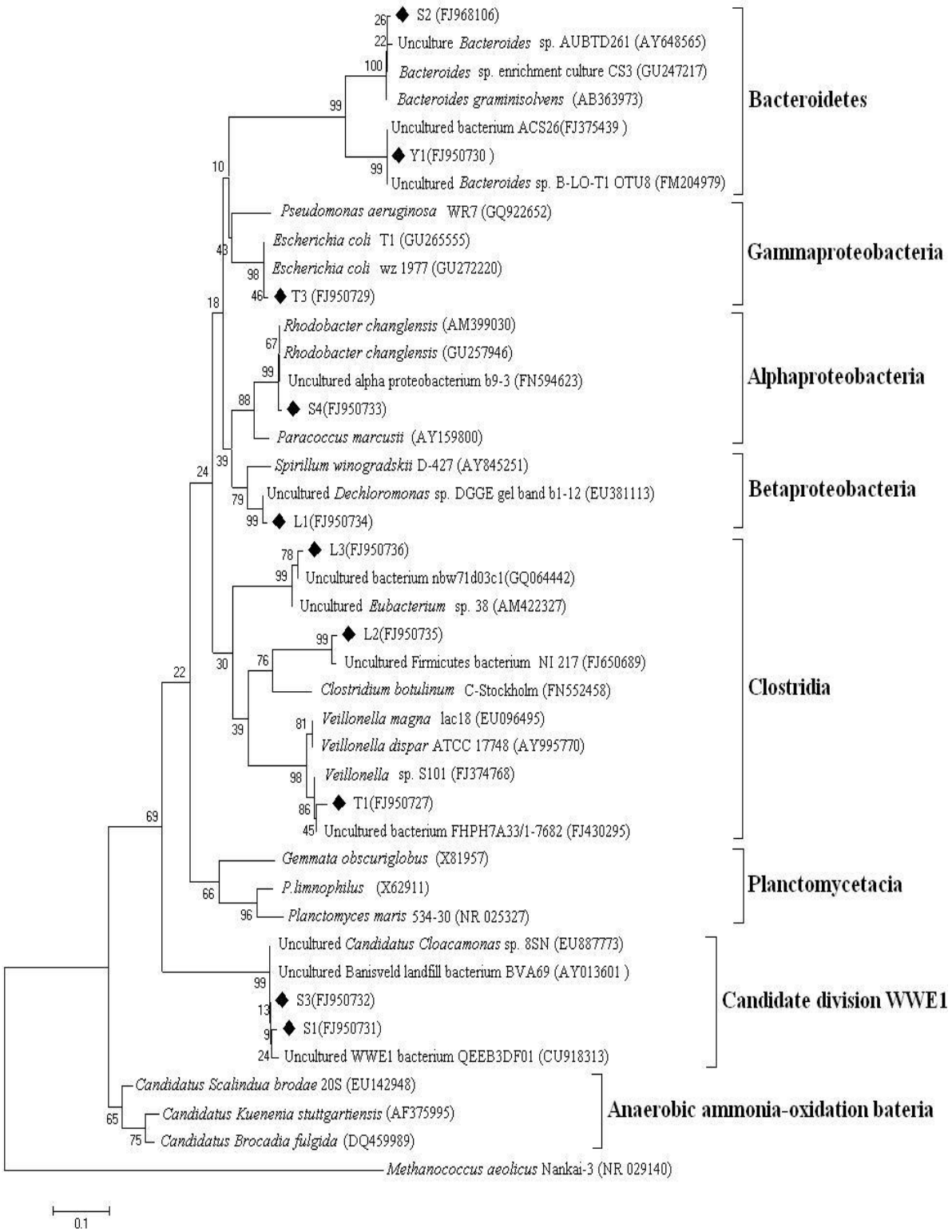

Figure 4. Phylogenetic tree constructed by NJ method and showing the affiliations of 16S rRNA gene sequences recovered from the wastewater treatment units of plant IF and M.

of clones. Further confirming work is under conduction using the method of 16S rDNA cloning and sequencing (Figure 4).

The distribution and diversity of bacteria in different

wastewater treatment system were significantly related to the sewage constituents and the operating factors such as hydraulic retention time, dissolved oxygen concentration and the flow or sludge returning. On the DGGE gel conducted by using a planctomycete-like bacterial forward primer and a universal reverse primer, no band was related to planctomycete-like bacteria, while two bright sequences were clustered into the unclassified group of Candidate division sharing only 92% identity with the reported planctomycete clones. No anammox activity was detected in the sludge samples from both the wastewater treatment plants.

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