Proc. KSAM Fall Meeting
October 28, 2000, Chonju, Korea

Phylogenetic Analysis of Bacterial Diversity of Enhanced Biological Phosphorus Removal Activated Sludge by Isolation and Cloning of 16S rDNA

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SUMMARY

Bacterial community structure composing enhanced biological phosphorus removal (EBPR) activated sludge was analyzed phylogenetically by cloning 16S rDNA after direct DNA extraction. Then, this result was compared with 16S rDNA sequences of randomly isolated bacterial species. The results clearly showed that there are no coincidence between the sequences retrieved directly from activated sludge and those of isolated strains, suggesting that many important bacteria are hidden in activated sludge because of the difficulty in isolation and culture of them.

INTRODUCTION

Recently, use of enhanced biological phosphorus removal (EBPR) processes in wastewater treatment has received increased attention because EBPR processes are lower in sludge production and running cost than conventional chemical processes. However, EBPR processes are difficult to control and sometimes deteriorate in phosphorus removal performance. For stable operation, a better understanding of the microorganisms in the activated sludge in the EBPR process is required.

In these processes, polyphosphate accumulating bacteria (PABs) and glycogen accumulating bacteria (GABs) are playing the key role to show EBPR activity in anaerobic/aerobic activated sludge. Many researchers have been focussing on *Acinetobacter* species as polyphosphate accumulating bacteria (Fhus and Chen, 1975; Deinema *et al.*, 1980; Cloete *et al.*, 1985; Groenestijn *et al.*, 1989; Lötter and Pitman, 1992; Beacham *et al.*, 1992). However, the carbon and phosphorus transformation patterns of these bacteria are not consistent with those of EBPR sludge (Ohtake *et al.*, 1985; Groenestijn *et al.*, 1989). The number of *Acinetobacter* species evaluated by culture-independent analyses, such as a fluorescent antibody method and an in situ hybridization technique, is very small in EBPR processes (Cloete and Steyn, 1987; Wagner *et al.*, 1993; Wagner *et al.*, 1994). *Microlunatus phosphovorus* (Nakamura *et al*, 1995a) was isolated as a polyphosphate accumulating bacterium showing typical

phosphate release and uptake (Nakamura et al, 1995b). However, this bacterium is not also dominated in EBPR activated sludge (Kawaharasaki *et al*, 1998). Glycogen accumulating bacteria showing substrate uptake coupled with glycolysis under anaerobic conditions have not been isolated until now. Recently, 16S rDNA analysis by cloning has been attempted to make clear the bacterial community structure of EBPR activated sludge (Bond et al, 1999; Hasselmann et al, 1999; Crocetti et al, 2000). However, the nature of both bacterial groups is still very unclear because of the difficulty of isolation.

In this research, direct DNA extraction and analysis of 16S rDNA by cloning have been attempted to analyze bacterial community structure of EBPR activated sludge, and these results were compared with the sequences of randomly isolated bacterial species.

MATERIALS AND METHODS

Activated sludge used.

EBPR activated sludge cultured by synthetic wastewater containing acetate as a main substrate using 1.8L sequencing batch reactor was used for DNA extraction and isolation. The reactor was operated in a cycle with three distinct periods consisting of an anaerobic period (60 min), an aerobic period (90 min) and a period (30 min) for settling sludge and replacing 0.9 liter of the supernatant with fresh synthetic wastewater.

Characterization by cloning of 16S rDNAs

Fractionation of activated sludge bacteria

Activated sludge cultured by anaerobic/aerobic batch reactor with acetate medium was sonicated (50 W, 20 sec.) after homogenization. Dispersed activated sludge was then centrifuged at 700 rpm, for 5 min, and sediment fraction was washed twice by distilled water. This fraction contained clustered cell (CC). Supernatant was mixed and centrifuged at 1,000 rpm, for 10 min, and sediment fraction was obtained as large cell (LC) fraction. Supernatant of LC fraction was then centrifuged at 10,000 rpm for 10 min, and small cell (SC) fraction was obtained. These three fractions were used to next experiments.

DNA extraction

The separated fractions containing cells were collected by centrifugation, and suspended in buffer (10mM Tris-HCl, pH7.5. 50mM EDTA, 0.5M NaCl). The Cell lysis procedure was based on the

method of Tsai et al. (1991) with some modifications (Ohkuma and Kudo, 1996). DNAs were extracted from lysates using a SepaGene Kit (Sanko Junyaku, Japan).

Amplification of 16S rDNA and cloning

16S rDNA fragments were amplified by PCR using the universal primers, Forward 5'-AGAGTTTGATCATGGCTCAG-3' and Reverse 5'-GGCTACCTTGTTACGACTT-3' (Weisburg et al, 1991). Cloning was performed using a SureClone ligation Kit (Pharmacia, Sweden).

Sequencing and phylogenetic analysis

Inserted 16S rDNA sequences (1.5kb) were determined with ABI 377 automated DNA sequencer (ABI, USA) using two sequence primers, M13 (-21) and 530F (5'-GTGCCAGCMGCCGCGG-3', 514 to 529 in *E. coli* numbering system). Obtained 16S rDNA sequences were aligned by a Clustal W 1.6 program (Tompson et al, 1994), and phylogenetic trees were constructed by the Neighbor-Joining method (Saito and Nei, 1987) with a MEGA.

Isolation of bacteria

LC bacterial fraction centrifuged at 1,000 rpm was used as an inocullum because this fraction is assumed to contain large cells like GABs and dense cells like PABs. NM-1 medium (Nakamura et al, 1995a) containing glucose (0.5g/l), peptone (0.5g/l), glutamate (0.5g/l), yeast extract (0.5g/l) as substrates was used for plating isolation. Colonies appeared on agar plates were picked up depending on different colony size, color and shape randomly.

RESULTS AND DISCUSSION

Characterization by cloning of 16S rDNAs

The numbers of clone obtained from each fraction, cluster cell fraction (CC), large cell fraction (LC) and small cell fraction (SC) were 19 clones, 20 clones and 18 clones, respectively. Totally, 57 clones were retrieved, sequenced and analyzed. The length of determined sequence of each insert was approximately 1.1kb, which was equivalent to 70% of the entire 16S rRNA sequence.

The phylogenetic analysis revealed that all retrieved sequences were roughly divided into three taxonomic groups or classes (Fig. 1). Forty clones were positioned in Proteobacteria. Seven clones and eight clones fell into the *Cytophaga/Flexibacter/Bacteroides* group and Verrucomicrobiales, respectively. Two remaining sequences were classified as in the *Plamctomyces* and *Acidobacterium*

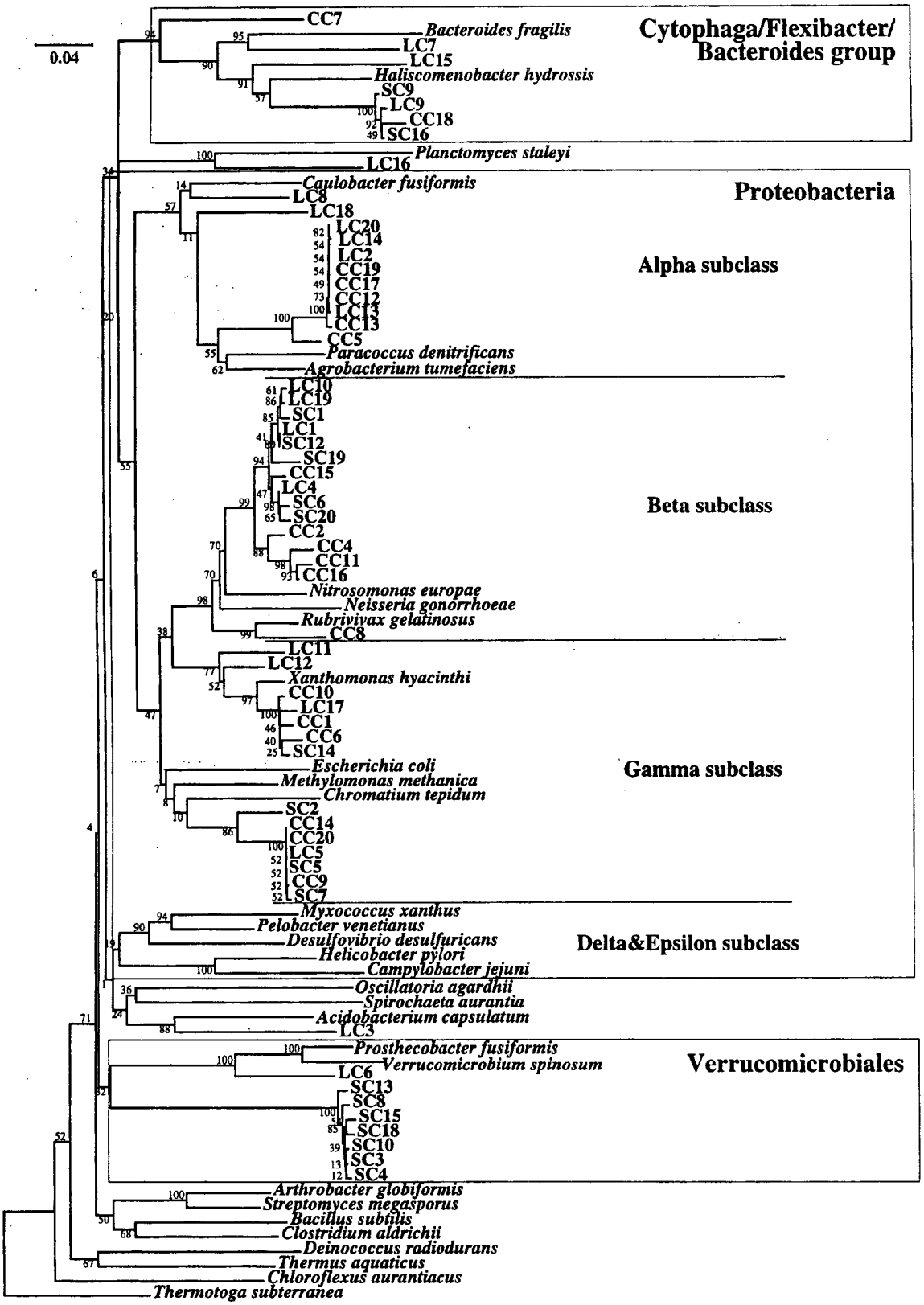

Fig. 1 Phylogenetic relationship between sequences directly cloned from activated sludge and the representative species in the *Bacteria*

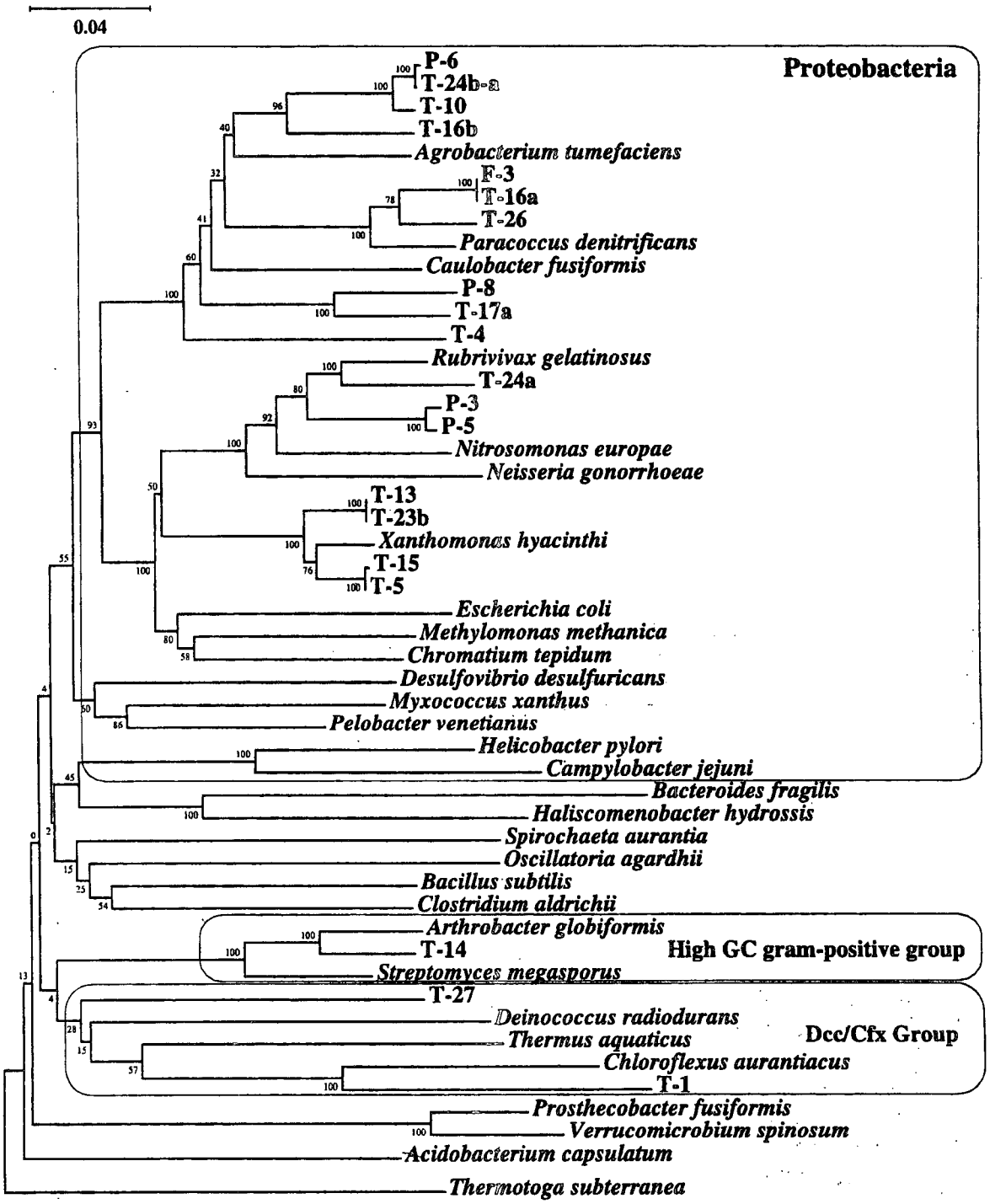

Fig. 2 Phylogenetic positions of newly isolated strains based on 16S rDNA sequence analysis

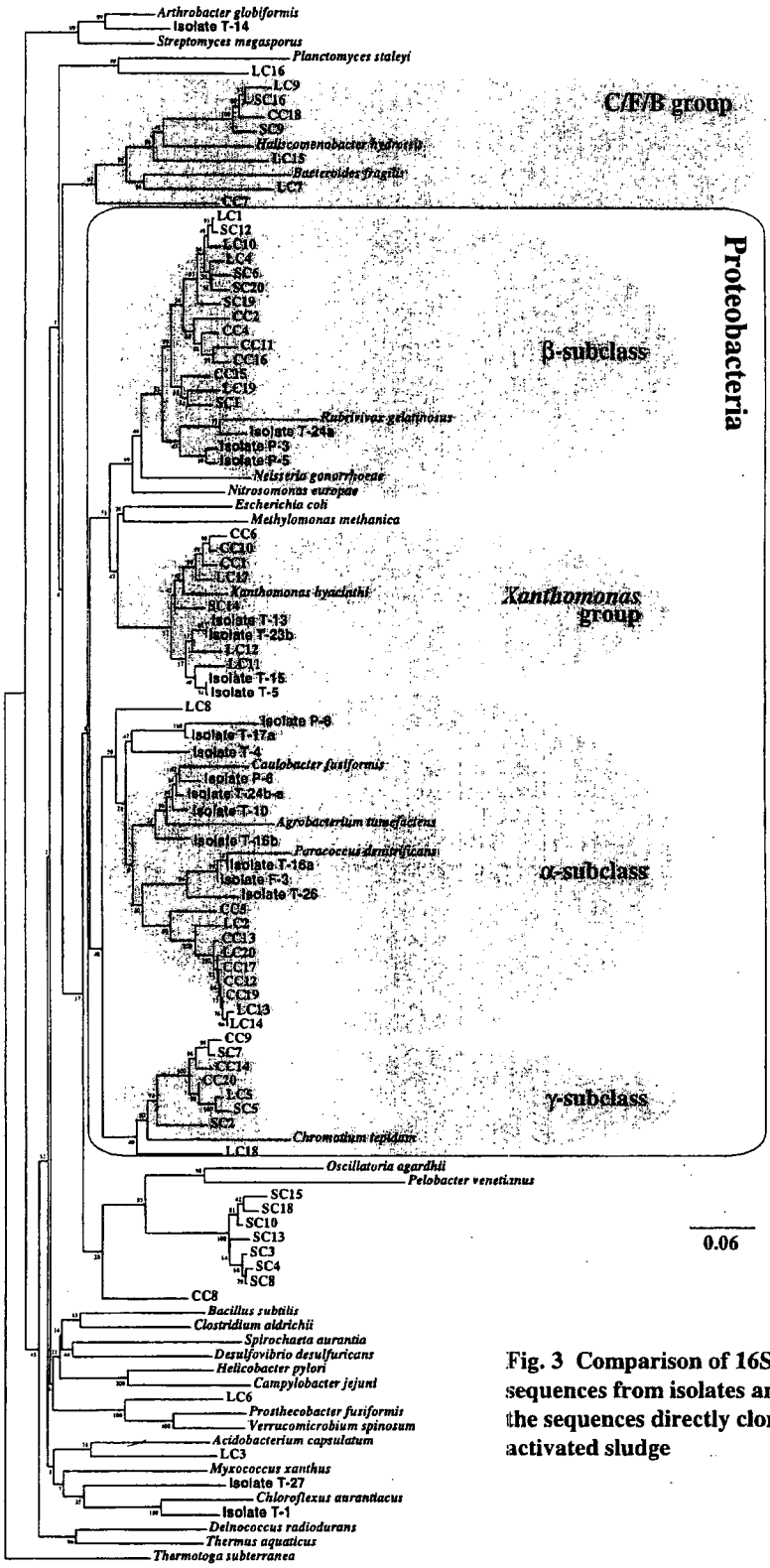

Fig. 3 Comparison of 16S rRNA sequences from isolates and the sequences directly cloned from activated sludge

groups, respectively.

Almost retrieved sequences were however relatively distant from any 16S rRNA sequences of all authentic species, therefore emerged as deeply branched linages in the phylogenetic tree. The seven retrieved sequences (SC3, SC4, SC8, SC10, SC13, SC15 and SC18) classified to Verrucomicrobiales were significantly distant from the authentic species in the group, *Verrucomicrobium spinosum* and *Prosthecobacter fusiformis*, suggesting the possibility to create a novel taxon (class) for the distinct SC group. In addition, no clone was classified to other taxa including Gram-positive bacteria.

Further phylogenetic analyses revealed that fourty clones belonging to Proteobacteria were distributed into alpha, beta and gamma subclass. In the alpha subclass, only one clone was classified to *Hyphomicrobium* group with a relatively high similarity, while other ten clones formed a distinct group which was deeply branched in the alpha subclass like *Caulobacter* group. In the beta subclass, almost clones (14 of 15 clone) were classified to *Rhodocyclus* group, and one clone belonged to *Sphaerotilus/Leptothrix/Ideonella/Rubrivivax* group. In gamma subclass, all clones were closed to *Xanthomonas* group.

Cloned sequences belonging to *Cytophaga/Flexibacter/Bacteroides* group, and *Rhodocyclus* group and *Xanthomonas* group of Proteobacteria were equally retrieved from each three fractions. However, cloned sequences of the deeply branching group in the alpha subclass (designated as *Caulobacter* group) and the distinct group closed to Verrucomicrobiales were mainly retrieved CC and LC fractions or SC fraction, respectively. The disproportion suggests that the former deeply branching group in the alpha subclass is due to form floc, but the later group closed to Verrucomicrobiales makes little contribution to floc-formation.

It is remarkable that almost cloned sequences retrieved from activated sludge were relatively distant from any 16S rRNA sequence of all authentic species.

Phylogenetic positions of the isolates

Twenty strains were newly isolated from LC bacterial fraction by a plating isolation method. While they had different colony size, color and shape, the cells of all isolates were morphologically rod-shaped. All isolates were able to grow by respiration.

The phylogenetic analysis based on 16S rDNA sequence comparison indicated that all isolates were differed from any authentic species, and which strongly suggested that the new taxon should be created for each isolates (Fig. 2). The phylogenetic positions of the isolates inferred from rRNA sequence comparison were scattered over the *Bacteria*. While only one isolate belonged to the high GC Gram-positive bacteria, almost isolates (17 strains) were related to the *Proteobacteria* and

distributed to three groups in this taxon, the alpha subclass (10 strains), beta subclass (3 strains) and *Xanthomonas* group (4 strains). Remained two isolates were akin to *Deinococcus/Chloroflexus* group (green non-sulfur bacteria), which was one of the deeply branched taxa

It is noteworthy that there are no coincidence between the sequences retrieved directly from activated sludge and those of isolated strains (Fig. 3). It suggest that many bacteria which have not been discovered yet consist in activated sludge, and are dominant over authentic species which can be isolated by usual plating method. Furthermore, the results of 16S rDNA analysis propose the possibility to create new distinct taxa for the unknown organisms hidden in activated sludge.

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