Identification of *Thiothrix unzii* in Two Distinct Ecosystems

Robin L. Brigmon,[*] Michelle Furlong[**] and William B. Whitman[**]

[*]Environmental Biotechnology Section, Savannah River Technology Center, Westinghouse Savannah River Company, Aiken, South Carolina, 29808,

[**]Department of Microbiology, University of Georgia, Athens.

LIST OF KEY WORDS

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[*]Corresponding Author

Environmental Biotechnology Section

Savannah River Technology Center

Westinghouse Savannah River Company

Bldg. 704-8T (TNX)

Aiken, SC 29808

Phone (803)-557-7719

Fax (803)-557-7223

EMAIL r03.brigmon@srs.gov

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Abstract

Molecular procedures were used to identify *Thiothrix* spp. in biofilms from sulfide-rich waters in two distinct Florida ecosystems. These *Thiothrix* spp.-containing biofilms at these sites have been consistently observed for over 10 years. Clonal libraries of biofilm 16S rDNA from each site contained rDNA sequences that were 99 to 99.5% similar to *Thiothrix* unzii.

Summary

The genus *Thiothrix* was created for ensheathed sulfur-oxidizing filamentous bacteria that deposit sulfur granules internally, attach to substrates, produce gliding gonidia, and form rosettes (Brigmon & DeRidder, 1998). *Thiothrix* spp. have been described as a component of biofilms on a variety of surfaces in sulfide-containing flowing water in natural systems (Brigmon et al., 1995) and wastewater treatment plants (Williams & Unz, 1989). The occurrence of *Thiothrix* as an ectosymbiont has been demonstrated for fresh water (Larkin et al., 1990) and salt-water (Polz et al., 1994) organisms. However, little is known about the molecular ecology of this genus. In flowing water with sulfide concentrations of at least 0.1 ppm, oxygen concentrations of less than 10% saturation and neutral pH, *Thiothrix* spp. attach to solid surfaces with holdfasts (Brigmon et al., 1995). In the springs and underwater limestone caves within the Floridan aquifer, visible white filamentous biofilms containing *Thiothrix* spp. have been observed as thin white mats or tufts with scattered distribution patterns on surfaces of rocks, sediments, and cave floors or filamentous masses in the water column (Brigmon et al., 1995).

In this study *Thiothrix* species were identified at two surficial sites with molecular techniques. We previously observed these biofilms to have similar physical appearances, physiological characteristics and sulfurous water sources. While there has been some anthropogenic influence on these aquatic systems, if left without treatment (i.e. chlorination), the same biofilm formation occurs

(Brigmon et al, 1997). This is the first reported identification of *Thiothrix* unzii in a natural aquatic system.

Materials and Methods

Sampling Sites.

Biofilm samples were obtained from two groundwater-fed systems in central and northern Florida including a spring at Orange Springs, Florida and a municipal water tank at Palatka, Florida. At both of these sites, visible colonies of white filamentous biofilms containing *Thiothrix* spp. have been documented for over 10 years (Brigmon et al. 1997).

Samples were collected from both sites on the same day with sterile forceps and immediately placed in sterile 50-ml centrifuge tubes. The Orange Springs samples were collected from the white filamentous biofilm covering the surface of rocks in the spring run at a depth of 0.5 m. The Palatka sample was collected from a biofilm-covered aerator pan. The water chemistry at both sampling sites was tested in the field for dissolved oxygen, pH, and hydrogen sulfide as previously described (Brigmon et al., 1997). Caution was used so that the tubes were opened under the water, biofilm mats immediately placed into the tubes, and capped before bringing to the surface. The tubes were stored in a cooler with ice and returned to the laboratory for processing.

The *Thiothrix* spp. in the biofilms were tentatively identified *in situ* for sampling by their typical white, rough, filamentous appearance (Larkin et al.,

1990). Initial microscopic examination of *Thiothrix* spp. distinguishing morphological characteristics including rosettes, filaments, gonidia, and sulfur granules was used for further presumptive identification (Williams and Unz, 1989). Confirmation of *Thiothrix* spp. in biofilm samples was made by monoclonal antibodies (MAb) specific for *Thiothrix* spp. with immunofluorescence as previously described (Brigmon et al., 1995).

Sample Phylogenetic Analysis

One milliliter of mat material was taken from each sample. This material was centrifuged at full speed in a microfuge for 5 min, washed with sterile distilled water and resuspended in 0.5 ml of sterile distilled water. The 16S ribosomal DNA in each sample was PCR-amplified using Ready-To-Go PCR Beads (Pharmacia Biotech, Piscataway, NJ). The bacterial primer 27f, 5'-AGA GTT TGA TCM TGG CTC AG-3' (Lane, 1991) and the universal primer 1392r, 5'-ACG GGC GGT GTG TRC-3' (Lane, 1991) were used at a concentration of 0.8 µM. For each sample, 2 µg of DNA, was added directly to a PCR reaction tube. The reaction mixtures were heated for 5 min at 94 °C, which lysed the cells. This lysate was used as a source of DNA without further purification. The denaturation, elongation, and annealing conditions were 1 min at 94 °C, 2 minutes at 72 °C and 1 min at 61 °C. The PCR products were purified by electrophoresis on a 0.8% agarose gel and eluted from the gel with a using Prep-A-Gene DNA Purification System (Biorad, Hercules). The amplified DNA was then ligated using T4 ligase into vector pCR 2.0 (Invitrogen, Carlsbad) using the vendor's protocol and transformed into *E. coli* Top 10 cells (Ausuvel et al., 1999, Miller et

al., 1988). After ligation, approximately, 100 colonies containing PCR-amplified bacterial 16S rDNA inserts from each sample were found. PCR inserts were sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia. Primer 27f was used to sequence 3 bacterial clones from each sample. Approximately 700 base pairs from each clone were sequenced.

For FastA searches, approximately 500 nucleotides of the sequences were utilized to avoid ambiguous positions in some of the clones. Both GenBank and EMBL databases were searched. Sequence comparisons were performed with PHYLIP 3.5 (Felsenstein, 1993). Evolutionary distances were calculated using the Jukes-Cantor formula, and the Neighbor Joining algorithm was used to construct the phylogenetic trees. Bootstrap analysis was performed with 100 replicates.

Results and Discussion

The clonal library from each sample site contained one clone that was 99%-99.5% similar to *Thiothrix unzii.* These clones, TX1 and PL3, were 99.8% similar to each other, suggesting that they both represented the same or very similar species. These two clones branched from the clade of *Thiothrix* spp. and clustered most closely with *Thiothrix* unzii with bootstrap value of 100 (Figure 1 & 2,data not shown) T. unzii has previously been found associated with wastewater systems (Howarth et al., 1999), and this is the first report of this taxon in a natural spring. This result suggests that this group may be more widely

distributed than previously known. The four other clones sequenced from the libraries were not related to the *Thiothrix* group and were less than 85% similar to each other. Presumably, they represent heterotrophic bacteria associated with these biofilms. The *Thiothrix* species was abundant enough in both mats to be detected in the Tx and PL clone libraries after sequencing only 3 clones. Moreover, this result is consistent with the previous observation that *Thiothrix* spp. comprised 18% w/w of the biofilm in the Palatka municipal water storage tanks (Brigmon et al., 1997). Previous work at these two sites indicated morphological and immunological similarity of the biofilms at these two sites (Brigmon et al.,1995). The water chemistry of the groundwater analyzed in the field at sampling time were quite similar. For Orange Spring and Palatka the groundwater constituents were to determined to be respectively 1 and 1.5 ppm sulfide, 2 and 5 % dissolved oxygen, and 7.2 and 7.3 pH. These conditions are conducive to growth of *Thiothrix* spp.

Knowledge of the microbial ecology in these aquifers and associated biofilms is limited. By increasing our understanding of the geomicrobial ecology of these environments, valuable information will be gained on our aquatic resources. This is important to document as anthropogenic pressure on these aquifers increases. Molecular procedures were applied here successfully to identify *Thiothrix* spp. in biofilms in two distinct ecosystems. These *Thiothrix* spp.-containing biofilms have been documented for over 10 years by microscopic, microbiological, and immunologic techniques. This confirmation by phylogenetic methods verifies the ecological distribution of related organisms in surficial

systems supplied by geochemically similar groundwater (Brigmon et al., 1994, 1997). This is the first report of *T. unzii* in a natural system.

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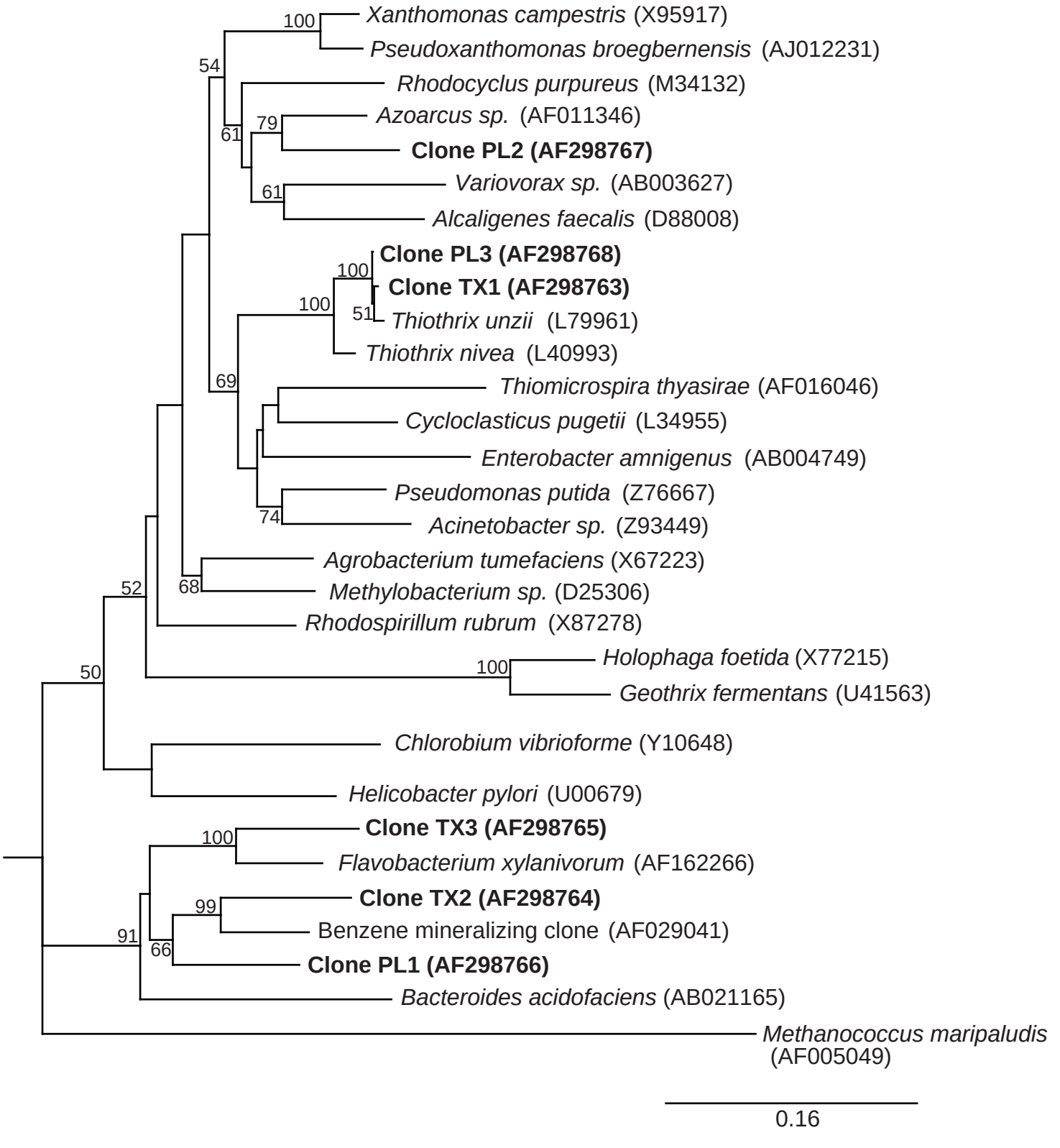
Figure 1. Phylogenetic tree generated by the neighbor-joining method from an alignment of 400 nucleotide positions showing the relationships between well PL and TX clones and described bacteria. PL and TX represent the samples from Palatka and Orange Springs, respectively. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence. This tree was rooted using a *Methanococcus maripaludis* 16S rDNA sequence. Bootstrap values greater than 50 are reported adjacent to each node.

Figure 2. Phylogenetic tree generated by the neighbor-joining method from an alignment of 659 nucleotide positions showing the relationships between the *Thiothrix* clones and described bacteria in the *Thiothrix* group. PL and TX represent the samples from Palatka and Orange Springs respectively. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence. This tree was rooted using a *Burkholderia caryophylli* 16S rDNA sequence. Bootstrap values greater than 50 are reported adjacent to each node.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Pseudoxanthomonas broegbernensis (AJ012231)
54
Rhodocyclus purpureus (M34132)
Azoarcus sp. (AF011346)
61
79
Clone PL2 (AF298767)
Variovorax sp. (AB003627)
61
Alcaligenes faecalis (D88008)
Clone PL3 (AF298768)
100
Clone TX1 (AF298763)
100
51
Thiothrix unzii (L79961)
Thiothrix nivea (L40993)
69
Thiomicrospira thyasirae (AF016046)
Cycloclasticus pugetii (L34955)
Enterobacter amnigenus (AB004749)
Pseudomonas putida (Z76667)
74
Acinetobacter sp. (Z93449)
Agrobacterium tumefaciens (X67223)
52
68
Methylobacterium sp. (D25306)
Rhodospirillum rubrum (X87278)
Holophaga foetida (X77215)
50
100
Geothrix fermentans (U41563)
Chlorobium vibrioforme (Y10648)
Helicobacter pylori (U00679)
Clone TX3 (AF298765)
100
Flavobacterium xylanivorum (AF162266)
Clone TX2 (AF298764)
99
Benzene mineralizing clone (AF029041)
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Clone PL1 (AF298766)
Bacteroides acidofaciens (AB021165)
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(AF005049)
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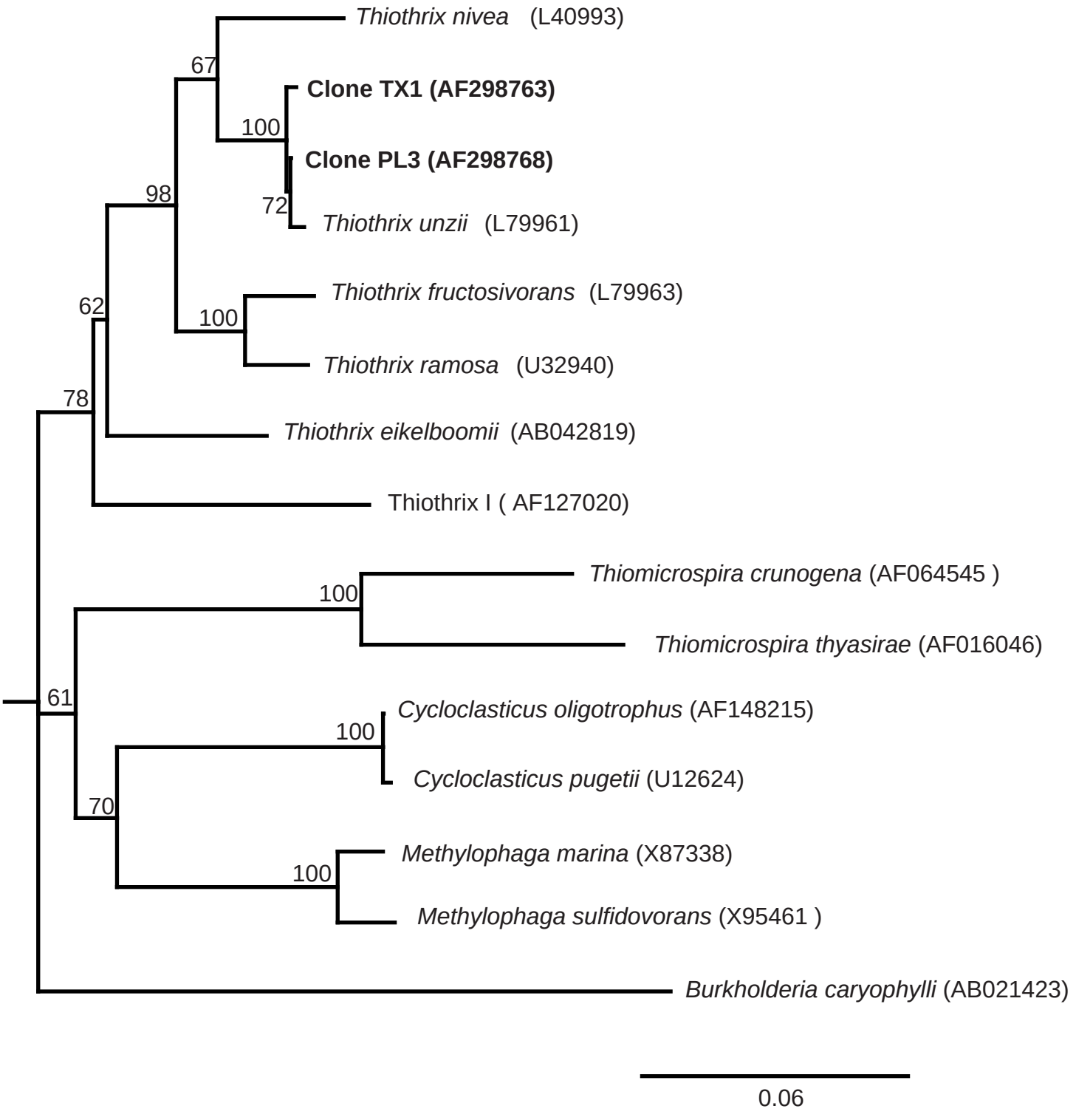
Thiothrix nivea (L40993)
67
Clone TX1 (AF298763)
100
Clone PL3 (AF298768)
98
72
Thiothrix unzii (L79961)
Thiothrix fructosivorans (L79963)
62
100
Thiothrix ramosa (U32940)
78
Thiothrix eikelboomii (AB042819)
Thiothrix I (AF127020)
Thiomicrospira crunogena (AF064545)
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