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Development and Application of a Monoclonal Antibody against *Thiothrix* spp.†

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Historically, methods used to identify *Thiothrix* spp. in environmental samples have been inadequate because isolation and identification procedures are time-consuming and often fail to separate *Thiothrix* spp. from other filamentous microorganisms. We described a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) procedure which was used to identify *Thiothrix* spp. in wastewater, artesian springs, groundwater, and underwater subterranean samples. The ELISA utilized monoclonal antibody T3511 to a species-specific carbohydrate epitope of *Thiothrix* spp. No cross-reactions were observed among non-*Thiothrix* strains consisting of 12 species and nine genera. In field trials, the ELISA identified 100% of 20 biochemically and cytologically confirmed *Thiothrix* spp.-containing samples with no false positives. Indirect immunofluorescent microscopy utilizing T3511 was effective for wastewater samples but not for those from natural spring water because of background fluorescence in the latter. In addition, electron micrographs of *Thiothrix* spp. labeled with T3511–biotin–anti-mouse antibody–gold showed that epitope T3511 was intracellular both in laboratory strains and environmental isolates. The minimum level of detection of the ELISA was 0.10 μg/ml.

The genus *Thiothrix* was created for strains of bacteria that deposit sulfur internally when in the presence of sulfide, produce ensheathed filaments that may attach to substrates, produce gliding gonidia from the unattached end of the filament, and form rosettes (18, 28). However, because of difficulties in obtaining pure cultures (2, 5, 11), *Thiothrix* spp. have been isolated and characterized only recently (17, 19, 22, 23). The ecology and distribution of *Thiothrix* spp. and other sulfur-oxidizing bacteria have been reviewed (16, 19). In nature, *Thiothrix* spp. are typically associated with leaves, rocks, or algae in sulfide-containing flowing water (14, 15). Under favorable conditions, *Thiothrix* spp. may so dominate a particular habitat that it is nearly a monoculture (21).

In drip irrigation (10) and citrus drainage systems (9, 27), growth of *Thiothrix* spp. can cause biofouling through physical blockage of water pipes. This blockage in pipes, valves, and drains occurs typically in association with iron and sulfur bacteria which can form a sulfur slime (10). Detection of these slime-associated problems is difficult in these systems until blockage occurs. The source of blockage (biological or physical) can affect the operation, which in some cases may necessitate drilling a new well or replacing the entire emitter system (24). *Thiothrix* spp. have been observed in aerobic wastewater treatment systems, where they have frequently been associated with activated sludge bulking (8, 29, 30). Bulking interferes with activated sludge settling and can cause economic problems. *Thiothrix* spp. were observed to be present in 12 of 16 activated sludge samples examined and the dominant form in 5 of 6 samples which were in the bulking state (7).

Although methods to identify most of the problem filamentous microorganisms exist, the methods require skill and experience (12) and the visual methods are subjective since the age of the filaments can affect the typical structural characteristics and the growth conditions can alter morphology (25, 26). Thus, improved methods are needed to rapidly identify the filamentous microorganisms responsible for sludge bulking and biofouling. The development of reliable and easy-to-use methods should aid in the diagnosis and solution of filamentous bulking and biofouling problems.

The present report describes an enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IIF), and immunocytochemical procedures which use a *Thiothrix* spp.-specific monoclonal antibody (MAb), T3511, to identify low numbers of *Thiothrix* spp. in enrichment broths and directly from samples from wastewater treatment plants, springs, groundwater, and underground caves.

MATERIALS AND METHODS

Microbial culture procedures. *Thiothrix* sp. strains A1 (ATCC 49747), Q (ATCC 49748), and I (ATCC 49749), grown on LTH medium (30), were obtained from Kay Shuttleworth, Department of Civil Engineering, The Pennsylvania State University, University Park. *Thiothrix* species types 1 and 3, *Leucothrix* sp. strain N11, and type 021N strain N2, grown on I medium (6) with $Na_2S \cdot 9H_2O$ at 0.187 g/liter and lactate substituted for glucose, were obtained from Michael Richard, Department of Environmental Health, Colorado State University, Fort Collins. *Thiothrix nivea*, grown on LTH medium, was contributed by Michael McGlannan, Department of Biological Sciences, Florida International University, Miami. *Thiothrix* sp. strains TA and TP were isolated as described previously (3).

For assay and immunization procedures, pure bacterial cultures were centrifuged, resuspended in phosphate-buffered saline (PBS) with 1% formalin, and refrigerated at 4°C. After overnight refrigeration, the bacteria were washed three times with PBS and stored at 4°C. Quantification of bacterial protein was accomplished by the method of Lowry et al. (20), modified to eliminate interference by sulfide or elemental sulfur (31).

Environmental samples. The primary source of activated sludge samples was a contact stabilization process treating domestic and hospital wastewaters from the University of Florida. Additional samples were obtained from a complete mix process treating domestic and commercial wastewaters from the Main Street and Kanapaha plants, Gainesville, Fla. Environmental samples were collected from sites observed to have white filamentous tufts attached to surfaces in flowing water indicative of the growth of *Thiothrix* spp. (17, 21, 22). All environmental samples were collected in Florida at the following sites: Warm Mineral Springs, Sarasota County; Orange Springs cave and spring, Marion County; the City of Palatka water storage tanks, Putnam County; sulfur slime-clogged drip irrigation

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TABLE 1. Bacterial species tested by ELISA using MAb T3511

Bacterial species and strain	ELISA reaction
Thiothrix sp. strain A1 (ATCC 49747)	+
Thiothrix sp. strain I (ATCC 49749)	+
Thiothrix nivea	+
Thiothrix sp. strain Q (ATCC 49748)	+
Thiothrix sp. strain TH1	+
Thiothrix sp. strain TH3	+
Thiothrix sp. strain TP	+
Thiothrix sp. strain TA	+
Type 021N N2	−
Beggiatoa alba (ATCC 33554)	−
B. alba (ATCC 33556)	−
Leucothrix sp. strain N11	−
Citrobacter freundii (ATCC 8090)	−
Desulfovibrio desulfuricans (ATCC 29577)	−
Enterobacter aerogenes (ATCC 13048)	−
Escherichia coli (ATCC 25922)	−
Pseudomonas aeroginosa (ATCC 27853)	−
Zoogloea ramigera (ATCC 19544)	−
Z. ramigera (ATCC 19623)	−
Z. ramigera (ATCC 25935)	−

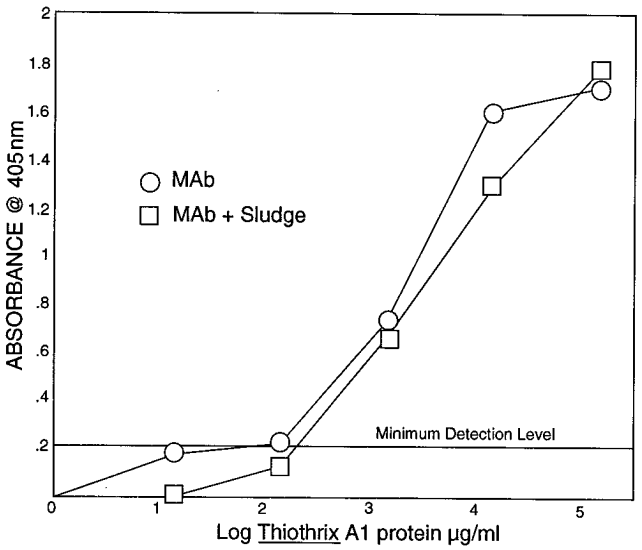

FIG. 1. Sensitivity of an ELISA for the detection of *Thiothrix* sp. strain A1 protein in pure culture and in 10% (wt/vol) sludge.

pipe (Florida Department of Agriculture, Polk County); and several underwater subterranean caves (Big Grungy Cave in Columbia County, Azure Blue Sink in Suwanee County, Devil's Ear Spring in Gilchrist County, Squirrel Chimney Sink in Alachua County, and Rossiter Spring in Hamilton County).

Samples were collected in sterile 50-ml polypropylene tubes (Costar Corp., Cambridge, Mass.), placed immediately on ice, and processed in the laboratory within 24 h. Water samples collected in sterile 1-liter polycarbonate containers (Corning Inc., Corning, N.Y.) at the time and site of sampling were used in LTH medium preparation for culture and enrichment procedures for *Thiothrix* spp. (3).

Production of MAb T3511. BALB/c mice were immunized initially by subcutaneous injection with 0.2 ml of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, Mo.) containing 100 μg of protein from *Thiothrix* sp. strain A1. This was repeated after 2 weeks. At week 4, the mice were injected intraperitoneally with 0.2 ml of Freund's incomplete adjuvant (Sigma) containing 100 μg of *Thiothrix* sp. strain A1 protein. The mice were immunized with the same quantity of *Thiothrix* sp. strain A1 in 0.2 ml of PBS at week 6 intravenously and at week 12 subcutaneously.

Splenocytes from immunized mice and myeloma cells (SP_2/0-AG14) were fused as described previously (4). After selection with hypoxanthine-aminopterin-thymidine medium, hybridoma supernatants from cultures showing significant growth after 21 days were tested for antibody production to *Thiothrix* sp. strain A1 by ELISA. Selected hybridomas were cloned by limited dilution, reidentified, cloned a second time, and retested by ELISA. Hybridoma T3511, which produced the highest titer in ELISA, was used in further studies. The isotype of MAb T3511 was determined with an ELISA kit (HyClone Laboratories, Inc., Logan, Utah). Hybridoma cells were passaged in pristane-primed BALB/c mice to produce specific antibody in ascitic fluid (4).

ELISA procedure. Formalinized washed *Thiothrix* sp. protein or other bacterial protein was diluted to 10 μg/ml in carbonate-bicarbonate buffer (pH 9.8), and 200 μl was added to 96-well Immulon 2 immunoassay plates (Dynatech, Chantilly, Va.). A positive control containing only *Thiothrix* sp. strain A1 and a negative control containing only *Leucothrix* sp. strain N11 were included on each plate. Plates were incubated for 16 h at 4°C. After adsorption of bacteria, ELISA plates were treated sequentially for 1 h with PBS containing 1% bovine serum albumin (PBSA; Hyclone Laboratories), 200 μl of antibody (antisera, hybridoma supernatant, or ascitic fluid preparation), and 200 μl of a 1:1,000 dilution in PBSA of affinity-purified alkaline phosphatase goat anti-mouse immunoglobulins (Organon Teknika, Malvern, Pa.). After incubation with each reagent, ELISA plates were then washed three times with PBS containing 0.05% Tween 20 (PBST; Sigma). Samples of hypoxanthine-aminopterin-thymidine medium, PBST, normal mouse serum, and media from supernatants of cultures of mouse myeloma cells (SP_2/0-AG14) were employed as negative controls. Antiserum from immunized mice used in the hybridoma protocol was used as a positive control. Bound conjugate was observed by addition of enzyme substrate solution (1 mg of *p*-nitrophenyl phosphate per ml in diethanolamine buffer; Sigma). The plates were read on a Titertek Multiskan plate reader (Flow Laboratories, McLean, Va.) at 405 nm after 30 min.

The ELISA procedure was performed on 20 bacteria, including eight *Thiothrix* strains, selected non-*Thiothrix* sp. filamentous bacteria, and various nonfilamentous bacteria found in the environment (i.e., wastewater) in which one would expect to find *Thiothrix* spp. All bacteria were tested at the same protein concentration (10 μg/ml) in ELISA buffer.

To test the lower threshold of assay sensitivity, pure cultures of the *Thiothrix* sp. strain A1 were grown and tested with the ELISA procedure at protein concentrations ranging between 0.00 and 144.3 μg/ml. The ability of the ELISA to detect *Thiothrix* spp. in complex samples was tested by adding *Thiothrix* sp. strain A1 to mixtures of 10% (wt/vol) sludge which were originally negative for *Thiothrix* spp.

Periodate oxidation was used in conjunction with the ELISA procedure to determine MAb specificity for carbohydrate determinants (32). Briefly, two plates were prepared for the ELISA with *Thiothrix* sp. strain A1 as described previously after incubation with PBSA. Plates were washed with 50 mM sodium acetate buffer (pH 4.5). One washed plate was then exposed to 200 μl of 10 mM sodium metaperiodate (Sigma) per well in 50 mM sodium acetate buffer (pH 4.5) for 1 h at 25°C in the dark. Following a brief rinse with 50 mM sodium acetate, both plates were then incubated with 200 μl of 50 mM sodium borohydride (Fisher Scientific, Fairlawn, N.J.) per well in PBS for 30 min at 25°C.

TABLE 2. ELISA testing for the presence of *Thiothrix* spp. in environmental samples using MAb T3511

Site	Presence of *Thiothrix* spp. determined by:	
	ELISA	Microbiological-cytological tests
Azure Blue Cave	+	+
Big Grungy Cave	+	+
Orange Springs Run	+	+
Orange Springs Cave	+	+
Palatka		
Aerator	+	+
Water	+	+
Gainesville sewage treatment plant	+	+
	+	+
	+	+
	+	+
Polk County	+	+
Rossiter Cave	+	+
University of Florida	+	+
	+	+
	+	+
Thiothrix sp. strain A1	+	+
Warm Mineral Springs	+	+
Kanapaha	−	−

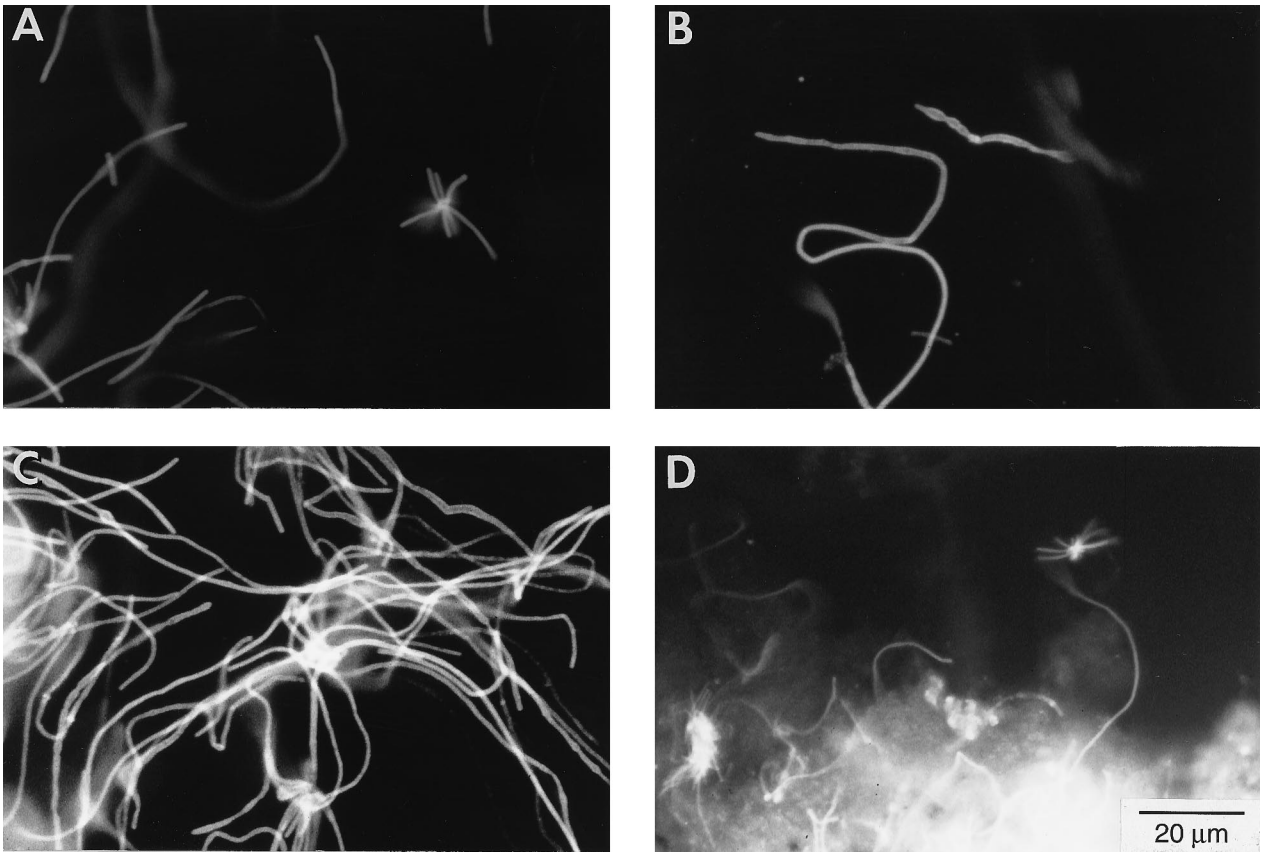

FIG. 2. IIF using MAb T3511. (A) *Thiothrix* sp. strain A1 labeled with MAb T3511 and FITC-labeled goat anti-mouse antibody; (B) *Thiothrix* sp. strain I labeled with MAb T3511 and FITC-labeled goat anti-mouse antibody; (C) *Thiothrix* sp. strain Q labeled with MAb T3511 and FITC-labeled goat anti-mouse antibody; (D) *Thiothrix* sp.-containing sludge labeled with MAb T3511 and FITC-labeled goat anti-mouse antibody from Gainesville Main Street sewage treatment plant. Bar, 20 μm (applies to all panels).

Environmental samples were washed three times with ELISA buffer and adjusted to pH 9.8 with 4 M NaOH immediately prior to the ELISA. Samples were then pipetted onto ELISA plates in duplicate. Controls included environmental samples with no MAb and samples with no secondary antibody on each plate. Positive controls with *Thiothrix* sp. strain A1 and negative controls with *Leucothrix* sp. strain N11 were also included on every plate. Samples thought to contain *Thiothrix* spp. were identified by the methods described by Jenkins et al. (12). Further tests to aid in the identification of *Thiothrix* spp. were the presence of poly-β-hydroxybutyrate granules as well as Gram and sheath stains (30). Samples were also enriched for 24 to 72 h with LTH medium at room temperature and observed by phase-contrast microscopy for formation of rosettes.

IIF. Pure cultures of *Thiothrix* sp. strains A1, Q, and I and environmental samples from activated sludge, Warm Mineral Springs, and Orange Springs were fixed for 30 min in PBS with 4% paraformaldehyde in microcentrifuge tubes (1.5 ml), subsequently washed three times with PBS, and then incubated for 10 min in 1 ml of PBS containing 10% goat serum. All samples were washed three times again with PBS and then incubated for 10 min with 1 ml of MAb T3511 or controls (PBSA or PBS). All samples were again washed three times and incubated for 30 min with secondary antibody, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (Sigma) diluted 1:50 in PBS. Samples were examined with a Nikon epifluorescent microscope. A drop of DABCO [1,4-diazobicyclo-(2,2,2)-octane; ICN Biochemicals, Costa Mesa, Calif.] in glycerol (Sigma) was added to samples on slides prior to microscopy to inhibit photofading of the FITC conjugate (13).

Immunocytochemistry. Bacteria were fixed for immunocytochemistry in 4.0% paraformaldehyde, 1.0% glutaraldehyde, and 50 mM lysine in PBS buffer at pH 7.2. Samples were dehydrated in 95% ethanol and embedded in Lowicryl K4M resin. Polymerization was done with UV light (280 nm) at −20°C, and the blocks were then thin sectioned. The sections were labeled with MAb T3511. The sections were first blocked with high-salt Tween buffer (0.2 M Tris HCl, 0.5 M NaCl, 0.1% Tween 20 [pH 7.2]), incubated with MAb T3511 diluted 1:1,000 in high-salt Tween buffer, and kept at 4°C overnight. Sections were then rinsed in high-salt Tween buffer for 10 min and two changes of PBS for 10 min each. After incubation for 1 h at room temperature, the sections were incubated with goat anti-mouse antibody conjugated to 18-nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) which was diluted 1:20 in PBS. Sections were then rinsed twice for 10 min in PBS and twice in distilled water for 10 min. The sections were stained with 5% uranyl acetate and Reynolds lead citrate and examined with a Hitachi 7000 electron microscope.

RESULTS AND DISCUSSION

Specificity and sensitivity assays. The results of an ELISA of MAb T3511 specificity of binding to a panel of select bacteria are shown in Table 1. A positive (+) result in the ELISA was an absorbance greater than 0.20 over controls. MAb T3511 was tested with 20 bacteria, including eight *Thiothrix* strains, four different filamentous bacteria, three *Zooglea ramigera* strains found in wastewater, and five other bacteria which could be in environmental samples. MAb T3511 was found to be of the immunoglobulin G subclass 2a (IgG2a) type comprising κ light chains specific for carbohydrate antigenic determinants only on the *Thiothrix* spp. tested. Results showed that positive reactions were limited to *Thiothrix* spp. The antigenic reactivity of MAb T3511 was destroyed when *Thiothrix* sp. strain A1-coated ELISA plates were subjected to sodium metaperiodate oxidation. Periodate treatment of carbohydrates opens the ring structure, and incubation with sodium borohydride blocks the free aldehyde groups. These results indicate that MAb T3511 recognizes carbohydrate-containing epitopes associated with *Thiothrix* sp. glycoproteins or glycolipids.

MAb T3511 was tested for sensitivity in the ELISA to the immunogen *Thiothrix* sp. strain A1. The results of these tests

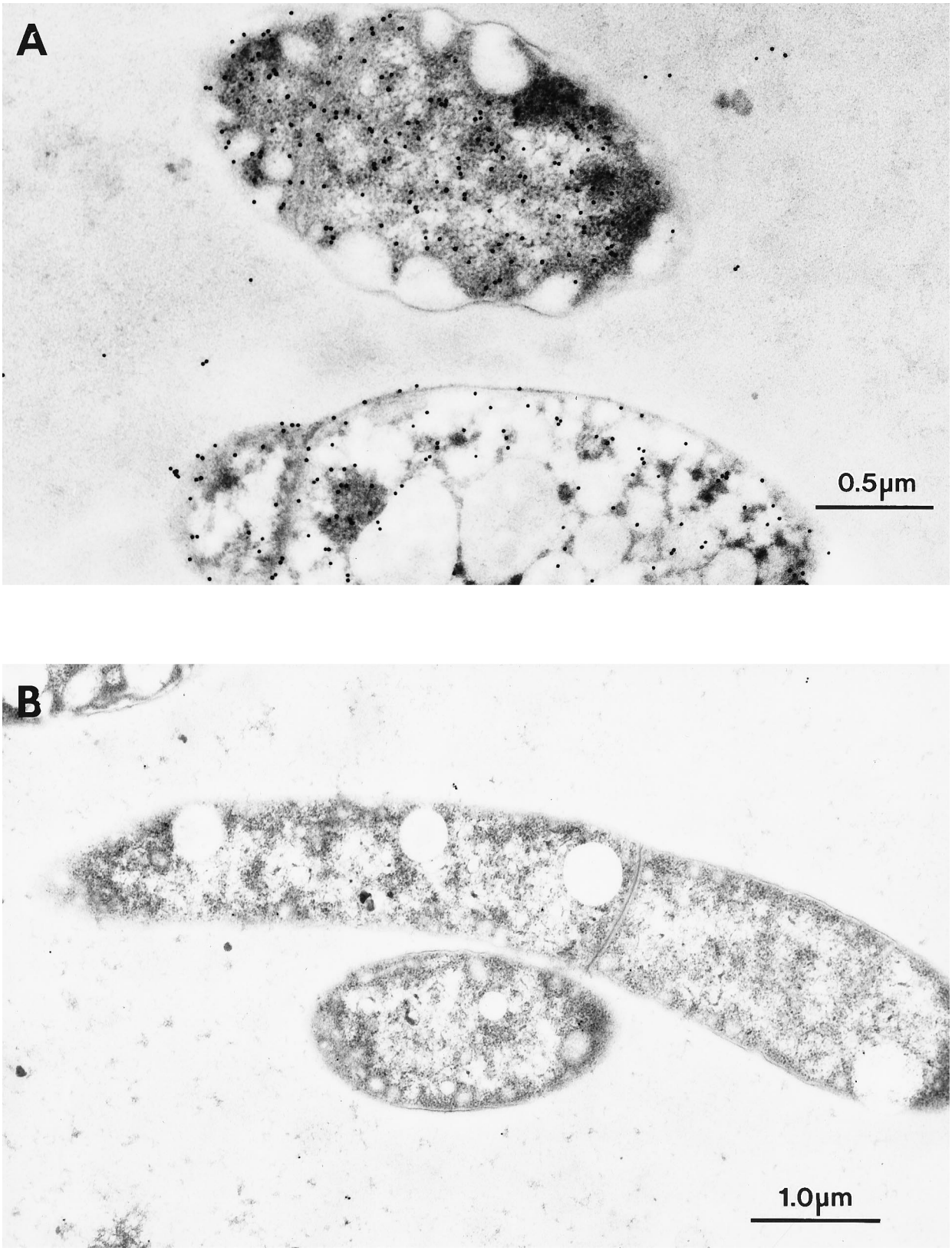

FIG. 3. (A) Thin section of *Thiothrix* sp. strain A1 labeled with MAb T3511 and IgG-gold complex. Bar, 0.5 μm. (B) Thin section of *Thiothrix* sp. strain A1 labeled with normal mouse serum and IgG-gold complex. Bar, 1.0 μm. (C) Thin section of *Thiothrix* sp. strain I labeled with MAb T3511 and IgG-gold complex. Bar, 0.2 μm. (D) Thin section of *Thiothrix* sp. strain Q labeled with MAb T3511 and IgG-gold complex. Bar, 0.5 μm.

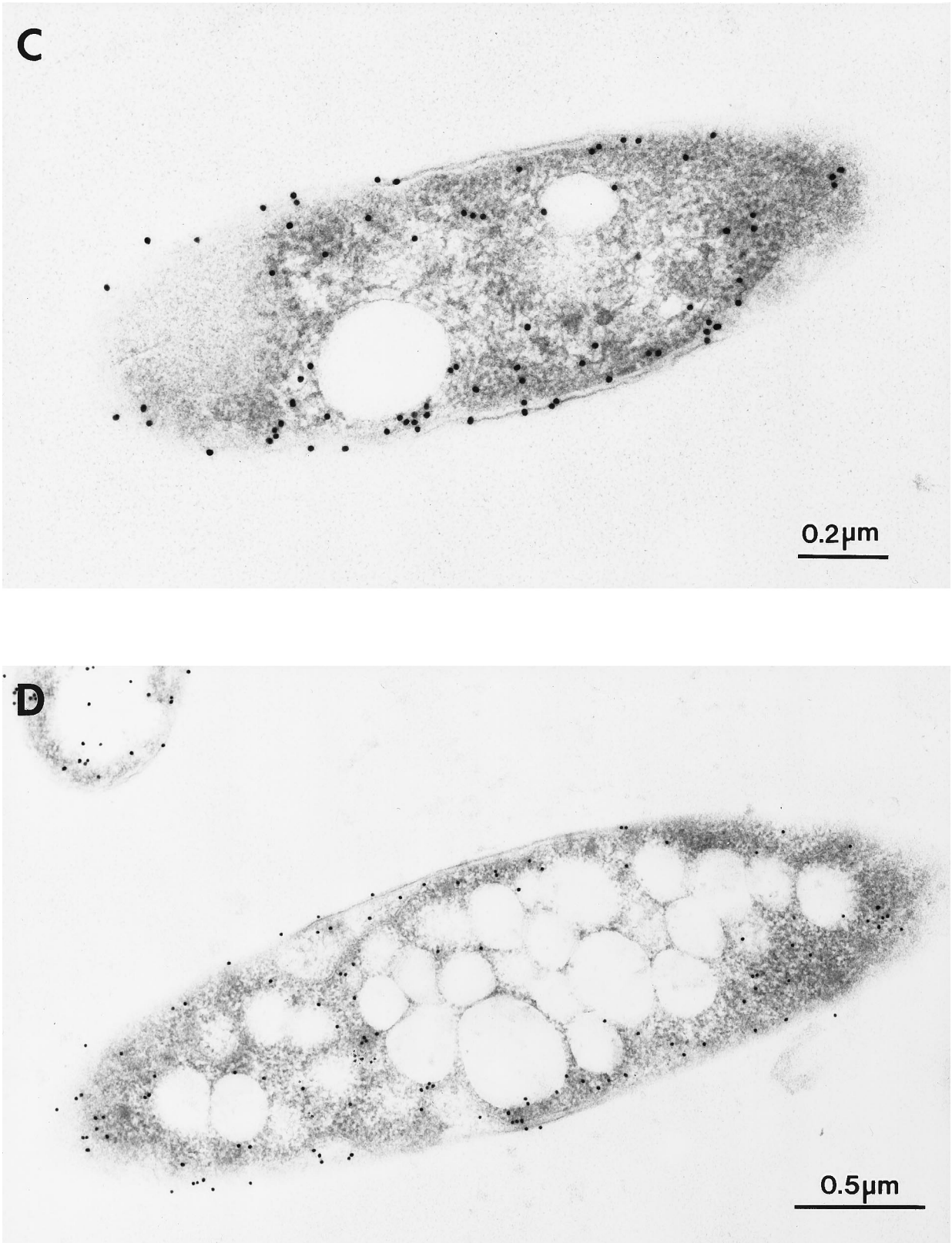

FIG. 3—*Continued.*

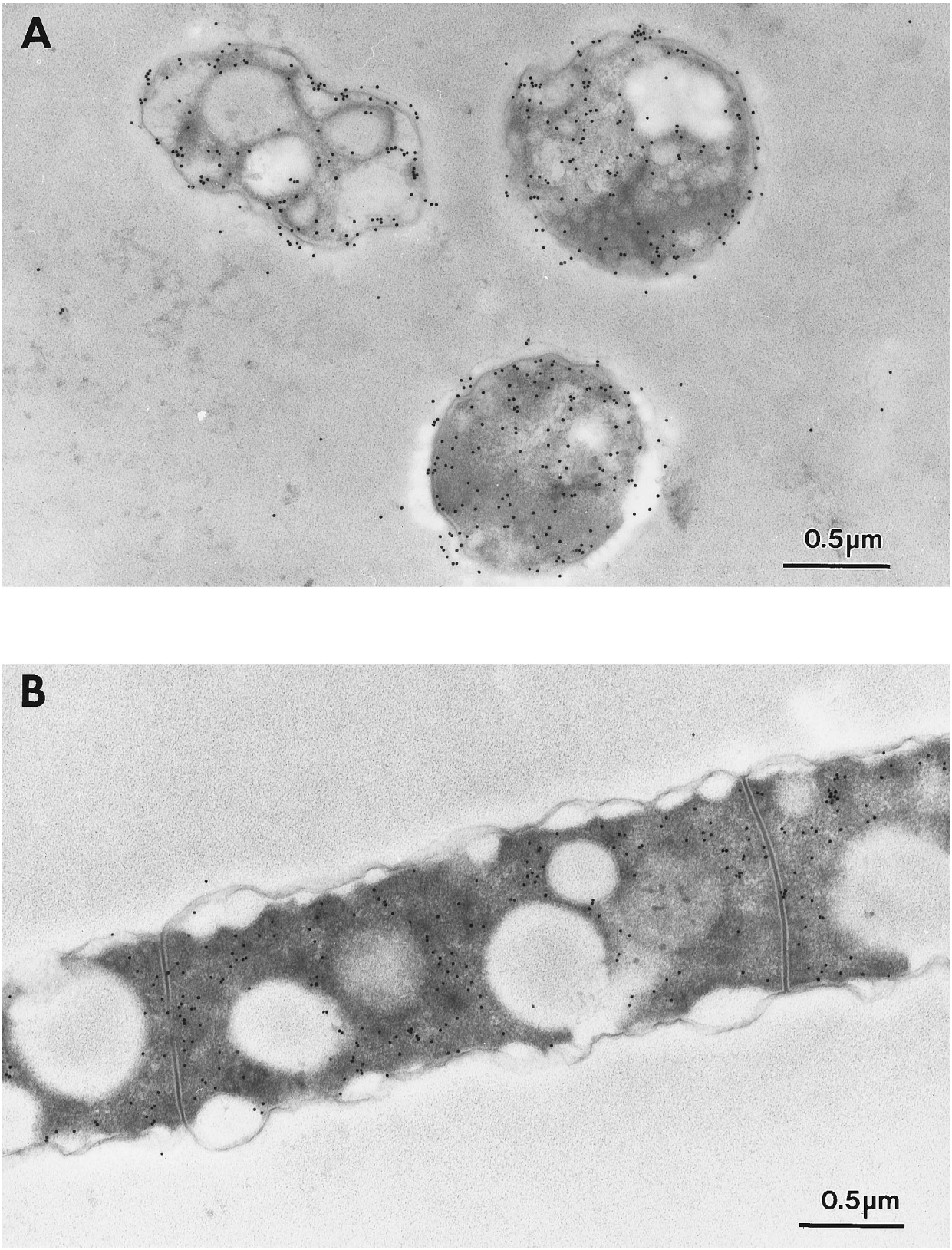

FIG. 4. (A) Thin cross section of *Thiothrix* sp. strain TA labeled with MAb T3511 and IgG-gold complex. Bar, 0.5 μm. (B) Thin longitudinal section of *Thiothrix* sp. strain TP labeled with MAb T3511 and IgG-gold complex. Bar, 0.5 μm.

are shown in Fig. 1. Results are from duplicate samples in the ELISA, as described previously. Logarithmic transformations were used to fit the curves. Absorbance was correlated ($P < 0.01$) with the concentration of *Thiothrix* sp. strain A1 protein in the ELISA. Although still significant ($P < 0.05$), lower correlations were observed between absorbance and the concentration of *Thiothrix* sp. strain A1 protein mixed 10% (wt/vol) in activated sludge which did not harbor *Thiothrix* spp. (Fig. 1). The minimum levels of detection were 0.10 and 0.50 μg/ml when testing *Thiothrix* sp. strain A1 in an axenic culture and in 10% (wt/vol) sludge, respectively (Fig. 1).

All samples which were positive for *Thiothrix* spp. by microbiological-cytological methodology were positive by the ELISA (Table 2). There were no false positives. The controls employed included sludge samples from the Gainesville Kanapaha treatment plant which were previously negative for *Thiothrix* spp.

Thiothrix spp. were not detected by ELISA, IIF, or microscopy methods in any samples obtained over a 12-month period from the Gainesville Kanapaha treatment plant, which never had any settling problems during the course of this study. *Thiothrix* spp. were detected, however, in 9 of 12 samples from the Gainesville Main Street plant and in 6 of 12 samples over the same 12-month period at the University of Florida plant by microbiological and immunological methods, including IIF and ELISA. Both of the plants in which *Thiothrix* spp. were detected did have settling problems.

IIF. Fluorescent photomicrographs of *Thiothrix* sp. strain A1 (Fig. 2A), strain I (Fig. 2B), and strain Q (Fig. 2C) with MAb T3511 at a 1:50 dilution are shown. Controls are negative. Only the Gainesville Main Street plant was ever sampled under bulking conditions during this study. Large quantities of *Thiothrix* spp. were observed by IIF when a load of oil had infiltrated the Gainesville Main Street plant, causing disruption of operation (Fig. 2D).

The *Thiothrix* spp. present in all spring samples were always associated with plant or alga material as well as other bacteria. All spring samples had a high level of background fluorescence. Additional washing of these samples and use of other blocking agents did not reduce the autofluorescence in these samples.

Immunocytochemistry. Examination of ultrathin sections of *Thiothrix* sp. strain A1 with MAb T3511 and the IgG-gold complex revealed that gold particles are distributed over the cytoplasm of cell periphery with no labeling of the sulfur inclusions or the poly-β-hydroxybutyrate granules in *Thiothrix* sp. strain A1 (Fig. 3A). When normal plasma was used instead of MAb T3511 (Fig. 3B), the cells of *Thiothrix* sp. strain A1 were practically free of any nonspecific binding of gold particles. *Thiothrix* sp. strains I (Fig. 3C) and Q (Fig. 3D) showed similar patterns of labeling with MAb T3511.

Cross sections of an isolate from an underwater cave, *Thiothrix* sp. strain TA (Fig. 4A), and longitudinal sections of an isolate from a water storage tank, *Thiothrix* sp. strain TP (Fig. 4B), also indicated cytoplasmic binding, with negligible labeling of granules or inclusion bodies.

A sensitive and specific MAb was developed and calibrated with the ELISA. Initially, *Thiothrix* sp. strain A1 was selected as the immunizing agent because no other axenic filamentous culture was available for testing. *Leucothrix* sp. strain N11 was used as a negative control because it is also a filamentous bacterium which forms rosettes. Bacterial protein was the best means of quantifying ELISA sensitivity because the filamentous nature of *Thiothrix* spp. prevents the use of standard microbiological enumeration methods such as plate counts.

More samples positive for *Thiothrix* spp. were found at the Main Street plant, which, historically, has always had some problems with settling and compaction of sludge. No *Thiothrix* spp. were detected in any of the samples from Kanapaha, which had no settling problems. There was excellent agreement in detection of *Thiothrix* spp. by MAb and ELISA with the more conventional methods employed here. In all cases, if there was a positive observation by microscopic techniques, a positive determination was made by ELISA.

The primary purpose in the development of this MAb was to directly identify specific microorganisms in complex ecosystems. The IIF procedure employed was a detection procedure with direct observation of fluorescent antibody-labeled filaments. The IIF worked well to identify *Thiothrix* spp. in pure culture and wastewater samples. However, samples from Orange Springs and Warm Mineral Springs treated with IIF appear to have a high level of autofluorescence due to endogenous natural compounds (derived mainly from plant material but also from algae), which inhibited direct observation of *Thiothrix* spp. The high density of fluorescent pseudomonads in a Florida spring containing *Thiothrix* spp. has been described (5). Autofluorescence in environmental microbiology is a function of the sample composition and can interfere with epifluorescent microscopy depending on wavelength (1).

Previous investigators have reported difficulty in isolating and identifying *Thiothrix* spp. and *Thiothrix* spp.-like bacteria from activated sludge (7, 29) and natural waters (2, 5, 11). As reported by Williams and Unz (30), media made with low levels of single-carbon substrates and thiosulfate or sulfide can enhance recovery of *Thiothrix* spp. from mixed liquors and enrichment cultures. In this study, enrichment of *Thiothrix* spp. by using LTH medium made with spring water (3) was coupled with an immunological procedure for the detection of *Thiothrix* spp. in environmental samples. The ELISA utilizing MAb T3511 was rapid and accurate, and the results were comparable to those of other procedures for identification of *Thiothrix* spp. which are time-consuming (12). The sensitivity and specificity of this MAb coupled with the IIF and ELISA techniques might make them useful tools for monitoring activated sludge systems for *Thiothrix* spp.-related bulking problems.

Bulking could be treated more efficiently if methods for early identification of the causative organisms were available (12). The methods described here could be refined into a faster, simpler ELISA or slide agglutination test. In this manner, an assay for *Thiothrix* spp. might be performed in minutes with the same sensitivity and specificity as those described here.

Future work in this area would include further identification of the specific antigen or epitope with which the MAbs are reacting. It would then be possible to extract the antigen (e.g., lipopolysaccharide) and increase the sensitivity of the ELISA. In addition, the use of amplification techniques would enhance the assay sensitivity. Rapid identification tests will probably improve ecological investigations of *Thiothrix* spp. These advancements will reduce the need for many microbiological tests.

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