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Fluorescent Acid-Fast Microscopy for Measuring Phagocytosis of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* by *Tetrahymena pyriformis* and Their Intracellular Growth

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Fluorescent acid-fast microscopy (FAM) was used to enumerate intracellular *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* in the ciliated phagocytic protozoan *Tetrahymena pyriformis*. There was a linear relationship between FAM and colony counts of *M. avium* cells both from cultures and within protozoa. The Ziehl-Neelsen acid-fast stain could not be used to enumerate intracellular mycobacteria because uninfected protozoa contained acid-fast, bacterium-like particles. Starved, 7-day-old cultures of *T. pyriformis* transferred into fresh medium readily phagocytized *M. avium*, *M. intracellulare*, and *M. scrofulaceum*. Phagocytosis was rapid and reached a maximum in 30 min. *M. avium*, *M. intracellulare*, and *M. scrofulaceum* grew within *T. pyriformis*, increasing by factors of 4- to 40-fold after 5 days at 30°C. Intracellular *M. avium* numbers remained constant over a 25-day period of growth (by transfer) of *T. pyriformis*. Intracellular *M. avium* cells also survived protozoan encystment and germination. The growth and viability of *T. pyriformis* were not affected by mycobacterial infection. The results suggest that free-living phagocytic protozoa may be natural hosts and reservoirs for *M. avium*, *M. intracellulare*, and *M. scrofulaceum*.

Mycobacterium avium, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* (the MAIS group) are environmental, opportunistic pathogens of animals and humans (11, 43). *M. avium* infections are found in patients with AIDS (15), inherited immune deficiency (1), cancer (41), and immunosuppression associated with transplantation (35). *M. avium*, *M. intracellulare*, and (rarely) *M. scrofulaceum* infections are also found in immunocompetent individuals with predisposing lung conditions such as silicosis and black lung (43). *M. avium* infections have also been reported to occur in individuals with cystic fibrosis (20) or pulmonary alveolar proteinosis (42). *M. avium* and *M. intracellulare* infections have been observed in elderly women lacking any of the known risk factors for *M. avium* infection (31). *M. avium* (currently) and *M. scrofulaceum* (until 1980) cause cervical lymph node infections in young children with erupting teeth (44).

M. avium, *M. intracellulare*, and *M. scrofulaceum* are frequently recovered in high numbers from both natural and drinking waters (7, 9, 12, 14, 17, 39) and soil (5, 16, 21). DNA fingerprints of *M. avium* isolates from AIDS patients were identical to those of isolates recovered from water consumed by the patients (40). Further, DNA fingerprints of *M. avium* isolates from simian immunodeficiency virus-infected monkeys were identical to those of *M. avium* in the animals' drinking water (24).

Ciliated phagocytic protozoans (e.g., *Tetrahymena*) are found in natural and drinking waters throughout the world (10, 19). In fact, *Tetrahymena* and members of the MAIS group share a number of common habitats, including drinking water (7, 9, 10, 14, 19, 34, 39) and peat-rich, acidic waters (17, 21, 23). Because the habitats of protozoa and *M. avium*, *M. intracellulare*, and *M. scrofulaceum* overlap, phagocytic protozoa may play a role in mycobacterial ecology and epidemiology. *M. avium* and *M. intracellulare* are intracellular pathogens, whose cells survive phagocytosis and can grow in macrophages (8) and epithelial cells (4). Cells of *M. avium* are capable of growth following phagocytosis by *Acanthamoeba castellanii* (6) and *Acanthamoeba polyphaga* (36). Further, intracellular growth of *M. avium* strain MAC 101 in *Acanthamoeba castellii* increased *M. avium* virulence in the beige mouse (6).

Because of the widespread prevalence of ciliated protozoa and *M. avium*, *M. intracellulare*, and *M. scrofulaceum* in natural and drinking waters, we sought to determine whether cells of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* could grow and survive within cells of *Tetrahymena pyriformis*. *T. pyriformis* was chosen because it is an easily cultured phagocytic protozoan and its habitats overlap those of *M. avium*, *M. intracellulare*, and *M. scrofulaceum*. One long-term goal of this work will be to identify mycobacterial genes involved in intracellular growth and survival.

MATERIALS AND METHODS

Mycobacterial strains. *M. avium* strains A5 (from an AIDS patient) (3), MAC 101 and MAC 104 (from AIDS patients) (18), and 13S (aerosol) (29) were used in these studies. *M. intracellulare* strain TMC 1406^T (ATCC 13950) and *M. scrofulaceum* strain TMC 1323^T (ATCC 19981) were also used. For all of the mycobacterial strains, transparent colonial variants were used (28).

***Tetrahymena* strain.** *T. pyriformis* strain ATCC 30202 was employed in these experiments.

Growth and enumeration of mycobacteria. Single transparent colonies of each strain were inoculated into 2 ml of Middlebrook 7H9 broth medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.5% (vol/vol) glycerol and

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10% (vol/vol) oleic acid-albumin enrichment (30), contained in a 16- by 125-mm screw-cap tube. Inocula were grown for 7 days at 37°C without agitation and refrigerated. Mycobacterial cultures for infection were prepared by inoculating 9 ml of M7H9 broth with 1 ml of the inoculum and incubating cultures at 37°C with aeration (120 rpm) until they reached mid-log phase (7 days). Mycobacterial cells were harvested by centrifugation (5,000 × *g* for 5 min at 25°C), the supernatant medium was discarded, and the cells were washed twice and suspended in 10 ml of phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 [pH 7.4]). The suspension was then centrifuged at 1,300 × *g* for 5 min at 25°C to pellet large aggregates (i.e., >25 cells per aggregate). The supernatant, containing only single cells and small aggregates (i.e., 5 to 25 cells per aggregate), was used immediately for infection (37). *M. avium* strain A5 was also grown in autoclaved drinking water (assimilable organic carbon range, 500 to 750 μg/liter) for 30 days at room temperature.

The number of mycobacterial cells per milliliter of culture or suspension was measured as CFU on Middlebrook 7H10 agar medium (BBL Microbiology Systems) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin enrichment (30) or as cell counts using a Petroff-Hauser chamber with or without fluorescent acid-fast staining (see below). Intracellular mycobacterial cells were counted by fluorescent acid-fast microscopy (FAM) or as CFU following sodium dodecyl sulfate (SDS) lysis of *T. pyriformis* cells (6).

Growth and enumeration of *T. pyriformis*. Cultures of *T. pyriformis* were grown in 5 ml of ATCC broth medium 357 (American Type Culture Collection, Manassas, Va.) contained in 18- by 150-mm screw-cap tubes at 30°C with aeration by rotation at 16 rpm. Cultures (0.5 ml) were transferred into 4.5 ml of fresh medium weekly. The number of cells of infected *T. pyriformis* per milliliter was determined by using a Petroff-Hauser chamber.

Fluorescent acid-fast staining. To stain extra- and intracellular mycobacterial cells, 10-μl samples of a mycobacterial or infected or uninfected protozoan culture were spotted onto glass slides and dried for 5 min at 70°C. Slides were flooded with an auramine O-rhodamine B-phenol stain (1.5 g of auramine O [Sigma-Aldrich Co., St. Louis, Mo.], 0.75 g of rhodamine B [Matheson Coleman and Bell, Norwood, Ohio], 75 ml of glycerol, 10 ml of phenol, and 50 ml of distilled water) for 15 min. Slides were rinsed in chlorine-free water and flooded with acid alcohol (0.5 ml of concentrated HCl in 100 ml of 70% ethanol) for 2 min. The slides were rinsed with chlorine-free water and then flooded with a 1.0-mg/ml solution of potassium permanganate for 3 min, which reduced the fluorescence of *T. pyriformis* cells but still allowed the margins and subcellular structure of the protozoa to be discerned. Slides were examined using a Zeiss Axiophot compound microscope equipped with a 545-nm excitation filter and a 610-nm emission filter under a 100× objective lens.

Comparison of FAM and CFU counts. Mycobacterial FAM and CFU counts of cell suspensions used for infection and of infected, twice-washed *T. pyriformis* cells were compared for *M. avium* strain A5. Intracellular *M. avium* CFU were measured employing the antibiotic treatment and SDS-shear lysis procedure (6).

Effect of *T. pyriformis* culture age on mycobacterial phagocytosis. Samples of a *T. pyriformis* culture were removed immediately following inoculation and at daily intervals into fresh ATCC 357 medium. The number of *T. pyriformis* cells was counted at each sampling time, and log-phase *M. avium* cells were added to the samples of *T. pyriformis* culture at a multiplicity of infection (MOI) of 10 *M. avium* cells to 1 *T. pyriformis* cell. Following a 30-min incubation at 30°C, 10-μl samples were collected and spotted onto glass slides and intracellular *M. avium* cells were counted by FAM. Phagocytosis of *M. avium* was expressed as both the number of intracellular fluorescent cells per protozoan cell and the percentage of protozoa containing fluorescent mycobacteria.

Time required for phagocytosis. *T. pyriformis* was grown as described above, *M. avium* cells were added at an MOI of 10:1, and the infected culture was incubated at 30°C. Samples were collected immediately and after 30, 60, 90, and 120 min of incubation. Phagocytosis of *M. avium* was expressed as both the number of intracellular fluorescent cells per protozoan cell and the percentage of protozoa containing fluorescent mycobacteria.

Intracellular growth of *M. avium*, *M. scrofulaceum*, and *M. intracellulare* in *T. pyriformis*. To determine whether *M. avium*, *M. intracellulare*, and *M. scrofulaceum* cells grew in *T. pyriformis* cells following phagocytosis, the following experimental protocol was used. One milliliter of a 7-day *T. pyriformis* culture was used to inoculate 9 ml of the *Tetrahymena* medium, and log-phase mycobacterial cells were added. Because of the rapidity of phagocytosis, the infected suspension could be centrifuged immediately at 2,000 × *g* for 10 min at 25°C to pellet the protozoan cells. The pelleted protozoa were washed twice by removal of 90% of the supernatant medium to reduce the number of extracellular *M. avium* cells 100-fold and thereby reduce further phagocytosis. The twice-washed *T. pyriformis* suspensions were transferred to 125-ml flasks and incubated with gentle swirling (60 rpm) at 30°C. Samples of the infected culture were collected immediately; at 12, 24, and 36 h; and then once per day on days 2 to 7. The number of *T. pyriformis* cells per milliliter of culture was determined for each sample, as were the numbers of intracellular and extracellular fluorescent acid-fast cells. For each sample, between 50 and100 *T. pyriformis* cells were examined, and the results from three independent experiments were averaged. The results are reported as the number of *T. pyriformis* cells per milliliter of culture, the number of intracellular *M. avium* cells (as intracellular fluorescent cells) per protozoan cell, the percentage of protozoa containing fluorescent mycobacteria, and the total number of intracellular *M. avium* cells per milliliter of culture. In addition to the uninfected control, two additional controls were included. First, the number of intracellular fluorescent cells was monitored in a *T. pyriformis* culture infected with cells from a suspension of *M. avium* strain A5 that was boiled for 30 min. Second, to demonstrate the ability of the *T. pyriformis* cells to digest microorganisms, the *T. pyriformis* culture was infected with *Escherichia coli* strain C cells at an MOI of 10:1. The *E. coli* cells were grown in M9 medium containing 0.2% (wt/vol) glucose (27) and washed and suspended in water immediately before infection.

Effect of MOI on intracellular *M. avium* growth in *T. pyriformis*. Intracellular numbers of *M. avium* cells were measured as fluorescent counts in *T. pyriformis* cells as described above following phagocytosis for 30 min at MOIs (*M. avium* strain A5 to *T. pyriformis* cell) of 1:10, 1:1, and 10:1.

Persistence of *M. avium* during long-term infection of *T. pyriformis*. *M. avium* strain A5-infected *T. pyriformis* cells were collected after 5 days of incubation at 30°C. A sample was removed to measure *T. pyriformis* and intracellular *M. avium* (FAM) numbers. One milliliter of the 5-day-infected culture was transferred to 9 ml of fresh ATCC 357 medium, and incubation was continued for 5 days (total of 10 days of incubation). Sample removal and subculture every 5 days was continued for totals of 15, 20, and 25 days of incubation. For each sample, between 50 and 100 *T. pyriformis* cells were examined, and the results from two independent experiments were averaged. The results are reported as the (FAM) number of intracellular *M. avium* cells per *T. pyriformis* cell and as the percentage of *T. pyriformis* cells containing fluorescent mycobacteria.

***M. avium* numbers following *T. pyriformis* encystment and germination.** *M. avium* strain A5-infected *T. pyriformis* cells were collected after 7 days of incubation at 30°C and 14 days at room temperature. Samples were removed at 7 and 21 days to measure the numbers of vegetative and encysted *T. pyriformis* cells and of intracellular *M. avium* (determined by FAM) cells. On the 21st day the encysted culture was transferred to fresh ATCC 357 medium (0.2 ml of culture in 20 ml of medium) and incubated at 30°C to allow for cyst germination. The cyst culture was sampled immediately and at daily (or twice-daily) intervals, and the numbers of cysts and vegetative *T. pyriformis* cells and intracellular *M. avium* cells (as FAM counts and CFU) were measured. For each sample, between 50 and 100 *T. pyriformis* cells were examined, and the results from three independent experiments were averaged.

Consequences of *M. avium* infection for *T. pyriformis* encystment and germination. *M. avium*-infected and noninfected *T. pyriformis* cultures were incubated for 5 days at 30°C. A sample was removed from both cultures, and intracellular FAM and protozoan numbers were measured. The 5-day cultures were then incubated at room temperature, and the numbers of *T. pyriformis* cysts and vegetative cells were counted daily. After 14 days of incubation, all *T. pyriformis* cells were encysted; 0.2 ml of each encysted culture was transferred to 20 ml of ATCC 357 medium and incubated at 30°C, and samples were collected at hourly intervals to enumerate cysts and vegetative (i.e., germinated) cells using a Petroff-Hauser counting chamber.

RESULTS

FAM. To avoid the use of antimycobacterial antibiotics (6) that might affect protozoan metabolism and behavior, we sought another method for enumerating intracellular mycobacteria. Acid-fast, cell-like particles were observed in uninfected *T. pyriformis* cells using the Ziehl-Neelsen stain. As a consequence, an auramine O-rhodamine B stain (38) was evaluated for enumeration of intracellular mycobacteria. Using that method, intact mycobacteria from cultures or suspensions of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* appeared as bright orange cells against a dark background. Uninfected *T. pyriformis* cells did not contain any fluorescent intracellular, mycobacterial cell-like particles. To provide a dark background with the outline of the *T. pyriformis* cells visible, a 3-min expo-

sure to potassium permanganate was found to be sufficient to reduce the background fluorescence of *T. pyriformis* without reducing the fluorescence of mycobacterial cells. Intracellular and extracellular mycobacterial cells could be distinguished and counted in infected *T. pyriformis* cultures. Single cells and cells in small aggregates (i.e., <15 cells per aggregate) could be counted as distinct orange cells using FAM. Cells in aggregates with more than 15 cells were difficult to count accurately using FAM because of cell overlap. Aggregates of larger than 25 cells in the suspensions used for infection were not encountered because they were removed by the low-speed centrifugation (37). In the studies reported here, aggregates of >15 cells were not seen in infected protozoa.

Linear relationship between FAM and CFU counts. There was a linear relationship between FAM and CFU counts of *M. avium* A5 suspensions prepared for infection as described above over a range of 1×10^2 to 4×10^5 ($r = 0.73$ by linear regression). In all infection experiments, *T. pyriformis* cells were washed twice following exposure to the mycobacteria. The two separate washing steps reduced the number of extracellular mycobacteria by a factor of approximately 100 such that there were 10 *T. pyriformis* cells per 1 extracellular mycobacterial cell. Reconstruction experiments established that the number of *M. avium* A5 CFU in supernatants of pelleted, twice-washed infected *T. pyriformis* cells was 0.08 to 0.15 the number of *T. pyriformis* cells. In addition to reducing the number of extracellular mycobacterial cells, care was taken to ensure that only intracellular fluorescent acid-fast cells were counted. Intracellular fluorescent bacilli could be distinguished from the few extracellular mycobacterial cells because the focal plane of the microscope was changed so that each *T. pyriformis* cell could be examined from top to bottom in a series of planes. Further, the margins of each cell could be identified to permit an unambiguous determination of whether a fluorescent mycobacterial cell was intracellular. We observed few fluorescent mycobacteria adherent to the outside margin of the *T. pyriformis* cells, suggesting that adherence is rapidly followed by phagocytosis. Intracellular FAM counts were compared to CFU of antibiotic-treated infected *T. pyriformis* cultures using the SDS-shear lysis procedure (6). Preliminary experiments confirmed that the SDS-shear lysis procedure (6) did not reduce mycobacterial colony counts and that greater than 99% of the *T. pyriformis* cells were lysed by that procedure. There was a linear relationship between intracellular FAM counts and CFU counts (6) for *M. avium* strain A5-infected *T. pyriformis* cultures ($r = 0.81$ by linear regression). The relationship between *M. avium* strain A5 CFU and FAM counts was lower for samples collected after 5 or more days of intracellular growth than for samples collected during the first 3 days, suggesting that mycobacterial cells were less able to form colonies after 5 days of intracellular growth.

***T. pyriformis* culture age influences phagocytosis of *M. avium* strain A5 cells.** Seven-day-old cultures of *T. pyriformis* are in stationary phase, and incubation after 7 days resulted in the appearance of cysts. Phagocytosis of *M. avium* strain A5, expressed as either the number of intracellular, fluorescent cells per protozoan cell or the percentage of protozoa containing fluorescent cells, was highest for the freshly transferred *T. pyriformis* cells (Table 1). The number of intracellular fluorescent cells per protozoan was 2.8 for the freshly transferred *T. pyriformis* culture (86% of *T. pyriformis* cells contained fluorescent cells). The number of *M. avium* cells per protozoan increased to 3.2 by day 1 and then was lower on succeeding days (Table 1). The data in Table 1 reflect averages from five independent *M. avium* and *T. pyriformis* cultures.

TABLE 1. Influence of *T. pyriformis* culture age on phagocytosis of *M. avium* strain A5

Age of *T. pyriformis* culture (days)	*M. avium*/*T. pyriformis* ratio (mean ± SD)[a]	% Infected *T. pyriformis* cells (mean ± SD)[b]
0	2.8 ± 0.21	86 ± 7
1	3.2 ± 0.4	95 ± 4
2	1.6 ± 0.2	76 ± 2.3
3	2.8 ± 0.42	87 ± 9.3
4	0.51 ± 0.2	38 ± 9.5
5	0.57 ± 0.06	45 ± 5
6	0.96 ± 0.05	60.3 ± 3.5
7	0.74 ± 0.15	49 ± 3

[a] Number of intracellular fluorescent bacteria per *T. pyriformis* cell.
[b] Percentage of *T. pyriformis* cells with at least one intracellular fluorescent bacterium.

Phagocytosis of *M. avium* strain A5 by *T. pyriformis* is rapid. Once it was shown that freshly transferred cultures of 7-day-old *T. pyriformis* had the highest rates of phagocytosis of *M. avium* strain A5, the length of time required for phagocytosis was determined. Phagocytosis appeared to occur rapidly (Table 2), and significant numbers of *M. avium* strain A5 cells were taken up by *T. pyriformis* cells collected immediately after infection. Evidently, phagocytosis occurred within the time required for the drying of the 10-μl drops on the slide (i.e., approximately 10 min). No other processing steps were required, because intracellular fluorescent mycobacterial cells could be easily distinguished by microscopy. There was no significant difference in the number of intracellular fluorescent cells per *T. pyriformis* cell whether the mycobacteria and protozoa were mixed and sampled immediately or after 30 min or longer periods of time (e.g., 120 min) (Table 2). The values in Table 2 reflect averages from three independent infections.

Intracellular growth of *M. avium*, *M. scrofulaceum*, and *M. intracellulare* in *T. pyriformis*. To determine whether mycobacteria could grow in *T. pyriformis* following phagocytosis, the following infection parameters were selected based on the previous results (Tables 1 and 2) and convenience. Freshly transferred *T. pyriformis* cells were infected at an MOI of 10 mycobacteria per protozoan and washed twice to reduce numbers of extracellular mycobacteria. Following resuspension of the

TABLE 2. Influence of exposure duration on phagocytosis of *M. avium* strain A5 by *T. pyriformis*

Exposure duration (min)	*M. avium*/*T. pyriformis* ratio (mean ± SD)[a]	% Infected *T. pyriformis* cells (mean ± SD)[b]
0	1.5 ± 0.6	67 ± 3.4
30	2.5 ± 0.6	81 ± 1.2
60	1.9 ± 0.3	77 ± 2.1
90	1.9 ± 0.4	81 ± 1.5
120	2.4 ± 0.2	91 ± 3.5

[a] Number of intracellular fluorescent bacteria per *T. pyriformis* cell.
[b] Percentage of *T. pyriformis* cells with at least one intracellular fluorescent bacterium.

TABLE 3. Intracellular growth of *M. avium* strain A5, *M. intracellulare* strain TMC 1406^T, and *M. scrofulaceum* strain TMC 1323^T in *T. pyriformis*

Incubation period (days)	*M. avium* A5			*M. intracellulare* TMC 1406^T			*M. scrofulaceum* TMC 1323^T		
	Mycobacteria/protozoan[a]	Protozoa/ml[b]	Mycobacteria/ml[c]	Mycobacteria/protozoan	Protozoa/ml	Mycobacteria/ml	Mycobacteria/Protozoan	Protozoa/ml	Mycobacteria/ml
0	2.5	1.6×10^4	4.0×10^4	1.3	1.2×10^4	1.6×10^4	3.1	2.0×10^4	6.3×10^4
1	1.2	1.3×10^5	1.6×10^5	5.5	5.8×10^4	3.2×10^5	1.3	1.2×10^5	1.6×10^5
3	3.6	2.2×10^5	7.9×10^5	1.5	5.3×10^5	8.0×10^5	1.0	2.5×10^5	2.5×10^5
5	4.6	1.1×10^5	5.0×10^5	1.5	4.2×10^5	6.3×10^5	1.1	2.9×10^5	3.2×10^5

[a] Number of intracellular fluorescent bacteria per *T. pyriformis* cell.
[b] Number of *T. pyriformis* cells per milliliter of culture.
[c] Number of intracellular fluorescent bacteria per milliliter of infected *T. pyriformis* culture.

twice-washed protozoa, a sample was collected for enumeration of protozoa and intracellular mycobacteria. The washing took approximately 30 min before the number of extracellular mycobacteria was reduced to prevent further phagocytosis. Therefore, the value for the number of *M. avium* strain A5 cells per protozoan on day 0 was equal to the value obtained after 30 min of phagocytosis (Table 2). Samples were collected twice daily to 7 days, and the number of intracellular fluorescent acid-fast cells per protozoan and the total number of fluorescent acid-fast cells per milliliter of culture were counted. In all experiments, fewer than five extracellular fluorescent acid-fast cells were observed per 50 to 100 protozoa counted. Thus, residual phagocytosis did not contribute to increases in intracellular acid-fast counts. The data from days 0, 1, 3, and 5 are presented in Table 3. The number of fluorescent acid-fast cells per milliliter of culture is the product of the number of fluorescent acid-fast cells per protozoan and the number of protozoa per milliliter of culture at each time point. It is important to point out that there was a five- to eightfold increase in *T. pyriformis* cells between days 0 and 1, and then the rate of increase fell (Table 3). If there was no growth of intracellular mycobacteria, the number of fluorescent acid-fast cells per protozoan should fall five- to eightfold between days 0 and 1. For *M. avium* strain A5, the number of fluorescent acid-fast cells per *T. pyriformis* cell fell from 2.5 on day 0 to 1.2 on day 1, but it then increased to 3.6 (a 1.4-fold increase) on day 3 and to 5.8 (a 2.3-fold increase) on day 7 (Table 3). In spite of the fall in the number of intracellular *M. avium* A5 cells per protozoan, their total number increased in the culture over 5 days because of the approximately 10-fold increase in *T. pyriformis* numbers. The number of intracellular *M. avium* A5 cells per milliliter of infected culture increased 4-fold between days 0 and 1 and then 10- to 20-fold by days 3 and 5 (Table 3). The increases between days 0 and 1 and days 1 and 3 were significant ($P < 0.001$ by the unpaired *t* test). By 7 days, the number of intracellular fluorescent cells per protozoan was 5.8 and the total number of *M. avium* A5 cells was 2.0×10^7 per ml of infected culture (data not shown). Thus, by both measures, *M. avium* strain A5 cells grew in *T. pyriformis* following phagocytosis. *M. intracellulare* strain TMC 1406^T also exhibited intracellular growth in *T. pyriformis* (Table 3). Like for *M. avium* strain A5, there was a fall in the number of intracellular mycobacteria per protozoan between days 0 and 1, but there was a 20-fold increase in total *M. intracellulare* numbers. By day 3, there was an increase in both the number of mycobacteria per protozoan and the total number of mycobacteria per milliliter of culture, and that measurement remained relatively constant at day 5 (Table 3). The increases between days 0 and 1 and days 1 and 3 were significant ($P < 0.001$ by the unpaired *t* test). *M. scrofulaceum* TMC 1323^T appeared to grow slowly in *T. pyriformis* (Table 3). The number of *M. scrofulaceum* cells per protozoan did not increase substantially after 3 and 5 days of incubation. However, there was modest intracellular growth of *M. scrofulaceum*, based on the fact that the total numbers at day 1 are 2.5-fold higher and the numbers of mycobacteria per protozoan are only 40% of the initial values in spite of a 10-fold increase in *T. pyriformis* cells. The increases between days 0 and 1 and days 1 and 3 were significant ($P < 0.003$ by the unpaired *t* test). Intracellular aggregates of fluorescent cells appeared in the *T. pyriformis* cells by the fifth day of incubation for both *M. avium* and *M. intracellulare*. However, the number of cells in the aggregates was less than 10, so they could be accurately counted.

To confirm that counts of intracellular fluorescent bacteria by FAM represented intracellular mycobacteria, FAM and CFU counts were measured in parallel. CFU measurements were performed on antibiotic-treated cultures using the SDS-shear lysis procedure (6). In addition, a number of different *M. avium* strains were included to describe the range of growth responses in this one species. The results, expressed as the FAM or CFU count at day 5 divided by the FAM or CFU count at day 0 (Table 4), illustrated that all of the *M. avium* strains and the representative *M. intracellulare* strain grew as determined by either measurement. The growth increases were higher for FAM counts than for CFU counts. There was a wide

TABLE 4. Comparison of 5-day growth increases of intracellular mycobacteria in *T. pyriformis* by FAM and colony counts

Species and strain	No. of expts	Increase in:	
		FAM count[a]	CFU[b]
M. avium			
A5	5	24	1.5
13S	2	19	4.2
MAC 101	2	73	5.2
MAC 104	2	8	3.0
M. intracellulare TMC 1406^T	4	35	2.1
M. scrofulaceum TMC 1323^T	4	4	0.4

[a] Number of intracellular fluorescent bacteria per milliliter of infected culture at day 5 divided by that at day 0.
[b] Number of CFU per milliliter of infected culture at day 5 divided by that at day 0.

TABLE 5. Influence of MOI on *M. avium* strain A5 phagocytosis by and growth in *T. pyriformis*

MOI	Day 0			Day 5			Increase in no. of *M. avium* cells/ml (fold)	Increase in CFU/ml (fold)
	M. avium/*T. pyriformis* ratio[a]	Mycobacteria/ml[b] (log)	CFU/ml[c] (log)	*M. avium*/*T. pyriformis* ratio	Mycobacteria/ml (log)	CFU/ml (log)		
10:1	2.4	4.4	4.4	4.5	5.8	4.6	24	1.5
1:1	0.11	3.0	4.0	0.41	4.9	4.1	79	1.6
1:10	0.07	3.0	2.9	0.17	4.3	3.2	22	1.8

[a] Number of intracellular fluorescent bacteria per *T. pyriformis* cell.
[b] Number of intracellular fluorescent bacteria per milliliter of infected *T. pyriformis* culture.
[c] Number of CFU per milliliter of infected *T. pyriformis* culture.

range of growth increases for the different *M. avium* strains (Table 4). For *M. scrofulaceum*, there was an increase in intracellular fluorescent bacteria (400%) by day 5, in agreement with the data in Table 3. However, there was a decrease in CFU (40%) after 5 days of incubation. Earlier we had noted that the correlation between *M. avium* strain A5 CFU and FAM counts was lower for samples collected after 5 days of intracellular growth than for samples collected during the first 3 days. Perhaps cells of *M. scrofulaceum* were even more sensitive to the possible stress provided by the intracellular protozoan environment.

Because *M. avium* is a waterborne opportunistic pathogen capable of growth in natural and drinking water (13), the phagocytosis and growth of water-grown *M. avium* strain A5 in *T. pyriformis* were measured. Because cell densities of water-grown MAIS cultures are 10-fold lower than those of medium-grown cultures (13), the MOI was only one *M. avium* strain A5 cell per *T. pyriformis* cell. Although the number of intracellular mycobacteria was low following phagocytosis (i.e., 0.08 fluorescent cell/protozoan), the value increased by day 5 (to 0.11 fluorescent cell/protozoan). Further, the number of intracellular fluorescent cells per milliliter of infected *T. pyriformis* culture increased from 4.0×10^3 (day 0) to 1.0×10^5 (day 5), a 25-fold increase.

M. avium strain A5 cells were killed by 30 min of boiling to determine whether they would be phagocytized and to monitor the number of intracellular fluorescent cells after phagocytosis. Boiled cells were less readily phagocytized by *T. pyriformis* (i.e., there was 1.0 intracellular fluorescent cell/protozoan) than were viable cells (Table 3). The lower value was not due to a decrease in MOI. The number of intracellular fluorescent cells fell to 0.07 (a 14-fold reduction) by 5 days of incubation of the infected culture. The lack of viability of *M. avium* strain A5 cells in either the boiled suspension or infected *T. pyriformis* was confirmed by the absence of colony formation on M7H10 agar.

To confirm that the *T. pyriformis* cells were capable of phagocytosis and digestion of bacteria, protozoa were infected with cells of *E. coli* strain C. In 7 days, the number of *E. coli* strain C CFU fell 300-fold (data not shown). Thus, the *T. pyriformis* cultures employed here were capable of digestion of normal protozoan prey bacteria.

Effect of MOI on intracellular *M. avium* growth. As suggested by the results of the infection of *T. pyriformis* with the water-grown *M. avium* cells, a lower MOI reduced the extent of phagocytosis (Table 5). However, intracellular growth did occur, whether measured as either the number of intracellular fluorescent cells or CFU (Table 5). To examine the effect of MOI on phagocytosis and intracellular growth, three MOIs were chosen. Those values represented the approximate ratios of mycobacteria to protozoa measured in common water types (7, 10, 19, 23, 39). The data in Table 5 are averages from four independent experiments at an MOI of 10:1 and of two independent experiments each at MOIs of 1:1 and 1:10. Although the numbers of intracellular fluorescent acid-fast cells were reduced at MOIs of 1:1 and 1:10, they were not strictly proportional. Two factors that likely contributed were the variation in the measurements (Table 2) and the possible ability of these motile protozoa to scavenge mycobacteria at a low MOI. At all three MOIs, *M. avium* A5 numbers increased between 0 and 5 days whether measured as number of intracellular fluorescent bacteria per protozoan or as CFU or number of intracellular fluorescent bacteria per milliliter of infected *T. pyriformis* culture (Table 5). The increase in CFU from day 0 to day 5 was lower than that shown by measuring the number of intracellular fluorescent bacteria. This is possibly due to the fact that the linear relationship between CFU and FAM counts is reduced upon prolonged exposure to the intracellular protozoan environment.

***M. avium* numbers are maintained during long-term infection in *T. pyriformis*.** If the reduced ability of mycobacterial cells to form colonies after 5 days of intracellular growth represents a reduced ability to grow, transfer of infected *T. pyriformis* to fresh medium (where protozoan growth resumes) might result in a decrease in mycobacterial numbers. To test this possibility, *M. avium*-infected cultures of *T. pyriformis* were grown for 5 days and sampled, and the infected protozoa were used as an inoculum (1:10 dilution) to initiate a culture in fresh medium. Four consecutive transfers were carried out on single cultures to permit measurement of intracellular fluorescent *M. avium* strain A5 numbers after 5, 10, 15, 20, and 25 days of incubation following phagocytosis. In this manner it was possible to characterize the behavior of intracellular *M. avium* strain A5 cells well beyond the encystment of *T. pyriformis* that initiated after 7 days of incubation at 30°C. The number of intracellular fluorescent bacteria (*M. avium* strain A5) per *T. pyriformis* cell was 4.0 on day 6 and remained close to that level on the ensuing sampling days. Thus, in spite of the repeated five- to eightfold increases in *T. pyriformis* numbers in the first day following transfer to fresh medium (Table 3), the number of mycobacterial cells remained constant. Long-term persistence of *M. avium* strain A5 cells in *T. pyriformis* was evidently not affected by the possible reduced ability of mycobacterial

cells recovered after 5 days of intracellular growth to form colonies.

***M. avium* survival following *T. pyriformis* encystment and germination.** By 21 days of incubation of uninfected or *M. avium* A5-infected *T. pyriformis* cells, greater than 99% of the protozoan cells had formed cysts. The encysted culture was used to inoculate fresh medium, and the number of intracellular fluorescent cells in vegetative (i.e., germinated) protozoa was counted. Germination was quite rapid, and by 1 day all (> 99%) of the cysts had germinated. The number of intracellular fluorescent bacteria per *T. pyriformis* vegetative cell was 2.2 ± 0.1 (average ± standard deviation) on day 21 and fell to 1.3 ± 0.1 on day 22, likely reflecting the rapid growth of *T. pyriformis* cells between days 0 and 1 following transfer. Colony counts confirmed the viability of the intracellular fluorescent acid-fast cells in the germinated *T. pyriformis* cysts.

Effect of *M. avium* infection on *T. pyriformis* growth, encystment, and germination. Because of the experimental design, the growth rates of uninfected (control) and mycobacterium-infected *T. pyriformis* could be measured. Infection by *M. avium*, *M. intracellulare*, or *M. scrofulaceum* neither increased nor decreased the number of *T. pyriformis* cells over the 5-day incubation period. Over the 5-day incubation period, the number of protozoa increased by an average of 10-fold. Further, greater than 98% of the *M. avium* strain A5-infected and uninfected protozoa collected at all sampling times excluded trypan blue.

No differences were observed in either the onset of encystment (measured as the frequency of cyst appearance after different lengths of incubation following infection) or germination (measured as the frequency of vegetative cells at different times following transfer of cysts to fresh medium) between uninfected and mycobacterium-infected *T. pyriformis* cultures. The first cysts appeared at approximately the same frequency in infected and uninfected *T. pyriformis* cultures after 5 days of incubation. The first vegetative cells occurred in cultures of infected and uninfected cysts approximately 3 to 5 h after transfer to fresh medium, and by 6 h 50% of the cysts had germinated.

DISCUSSION

In addition to demonstrating the utility of FAM for detection and measurement of the phagocytosis and growth of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* in the phagocytic ciliated protozoan *T. pyriformis*, these results have ramifications for understanding the epidemiology and ecology of environmental opportunistic mycobacteria. The results demonstrate that phagocytic, ciliated protozoa are likely reservoirs of the MAIS group in the environment.

FAM (38) can be used to accurately detect and enumerate intracellular mycobacteria in phagocytic protozoa. It is clearly superior to the Ziehl-Neelsen acid-fast stain for this application because of the absence of artifacts. Because fluorescent acid-fast intracellular mycobacteria could be detected in the waterborne phagocytic amoeba *A. polyphaga* as well as in *T. pyriformis*, FAM can be used with many species of protozoa and perhaps even animal macrophages. The number of fluorescent acid-fast bacteria correlated with the number of CFU for pure mycobacterial cultures (correlation coefficient = 0.75). This was not unexpected, because fluorescent counts were always higher than colony counts and mycobacterial particle counts are always higher than colony counts (26), especially for transparent colonial variants, such as were employed in this study. A similar correlation ($r = 0.81$) was found for comparison of numbers of intracellular fluorescent bacteria (FAM counts) and colonies recovered after SDS-shear lysis (CFU). Because of the many manipulations involved in the SDS-shear lysis procedure (6) and the tendency of mycobacteria to adhere to surfaces, it was expected that FAM counts would be higher than CFU counts. There was a wider difference between intracellular *M. avium* strain A5 FAM counts (higher) and CFU counts (lower) for samples collected after 5 or more days of intracellular growth than for samples collected during the first 3 days. That difference suggests that mycobacterial cells were less able to form colonies after 5 days of intracellular growth in *T. pyriformis*. However, that loss of colony-forming ability evidently did not reduce the ability of that strain to persist over four transfers, repeated 10-fold increases in protozoan numbers, and a total of 25 days of intracellular growth. Reduced colony formation may be a consequence of the transfer of mycobacteria from an intracellular environment with a reduced oxygen level to the oxygen- and oxygen metabolite-rich surface of an agar medium. As such, it might not be related to the ability of mycobacteria to persist in the intracellular protozoan environment.

After establishment of the utility of FAM for enumeration of intracellular mycobacteria, factors affecting the phagocytosis of mycobacteria by *T. pyriformis* were identified to establish standard conditions for infection. First, the growth characteristics of *T. pyriformis* limited our length of incubation of infected protozoa to 5 days to avoid problems of encystment. Culture medium inoculated with a 10-fold dilution of a 7-day culture of *T. pyriformis* readily phagocytized mycobacteria (Table 1). Phagocytosis occurred rapidly and reached a maximum within 30 min (Table 2). In fact, it was sufficient to simply mix mycobacteria and protozoa and collect a sample. Phagocytosis occurred during the time necessary to dry the 10-μl spots on the slides. Rapid phagocytosis is not unexpected, because the protozoa are starved and have entered stationary phase (19, 23). The ratio of mycobacteria to protozoan cells did influence the resulting number of intracellular mycobacteria immediately after phagocytosis (Table 5). The MOIs used were chosen because they reflect the ratios expected to be found in the aquatic habitats common to mycobacteria (7, 39) and *Tetrahymena* (10, 19, 23). Although the MOI influenced the number of intracellular mycobacteria immediately after the period of time allowed for phagocytosis, it did not influence the increase in number of intracellular mycobacteria after 5 days (Table 5). As a result of that phase of the study, conditions were chosen for measurements of growth of intracellular mycobacteria. *T. pyriformis* cultures inoculated with 7-day-old cultures were infected with 10 mycobacteria per protozoan, and immediately following mixing the suspension was washed twice and suspended in fresh *Tetrahymena* medium and a sample was removed for measurement of *T. pyriformis* and intracellular mycobacteria.

This study confirms that aquatic protozoa can serve as hosts for the growth of environmental, opportunistic mycobacteria. *M. avium* strain MAC 101 (serotype 1) grew in the phagocytic

amoeba *A. castellii* (6), and a serotype 4 isolate of *M. avium* grew in and survived encystment of *A. polyphaga* (36). The earliest report of mycobacterial growth in *A. castellanii* reported phagocytosis and "retention" of cells of individual isolates of *M. avium*, *Mycobacterium marinum*, *Mycobacterium ulcerans*, *Mycobacterium simiae*, and *Mycobacterium habana* (22). Thus, the list of suitable protozoan hosts for *M. avium* is now extended to the phagocytic, ciliated protozoan *T. pyriformis*. Although in the majority of infection experiments FAM was employed for enumerating mycobacteria, the increases are not artifacts of the method. First, FAM and CFU counts were correlated. Second, increases in FAM counts were confirmed by increases in CFU counts, albeit not to the same magnitude. Third, although boiled *M. avium* strain A5 cells were phagocytized, the number of intracellular fluorescent cells fell 14-fold, consistent with an absence of growth. Fourth, FAM counts increased in spite of rapid multiplication of *T. pyriformis* immediately following infection (days 0 to 1). Finally, it was possible to identify mycobacterial cells because of their unique morphology and to distinguish between intra- and extracellular mycobacterial cells by fluorescence microscopy. Thus, the FAM numbers were not inflated by inclusion of cell-like particles or extracellular mycobacterial cells. In all experiments, few if any extracellular fluorescent mycobacterial cells were observed following the washing procedure. Further, the mycobacteria grew very slowly in the *Tetrahymena* medium, with a generation time of approximately 2 days at 30°C. The growth of intracellular mycobacteria in *T. pyriformis* was not due to the inability of the protozoa to digest bacteria, because the number of *E. coli* cells fell 300-fold in 5 days.

The ability of intracellular mycobacteria to grow and increase in number in *T. pyriformis* is at variance with the well-known slow growth of *M. avium*, *M. intracellulare*, and *M. scrofulaceum*. This is especially problematic considering the 10-fold increase in the number of *T. pyriformis* cells in the day immediately following infection and transfer into fresh medium. If mycobacterial numbers can double in 1 day (26), there should have been a fivefold fall in the number of mycobacteria per protozoan. However, the data show only a twofold decrease, suggesting that mycobacteria are capable of higher rates of growth (e.g., two doublings per day). *M. avium* has been shown to grow at a rate of one doubling per 8 to 12 h immediately upon transfer into fresh mycobacterial medium (32). The growth rate fell to one doubling per 24 h after 1 day of incubation (32). The MAIS strains employed in this study share those growth characteristics. Those data are consistent with the ability of intracellular mycobacterial cell numbers to be maintained in spite of the 10-fold increase in *T. pyriformis* numbers within the first day after transfer to fresh medium.

To determine the range of values for intracellular growth of *M. avium*, the studies included *M. avium* strains of diverse origin (i.e., from patients and from the environment). Although all strains grew, the increases of numbers of intracellular fluorescent acid-fast cells were different (Table 4). We are intrigued by the possibility that the strains demonstrating the highest increases (i.e., strains A5 and MAC 101) are more pathogenic than the strains with lower increases (i.e., strains MAC 104 and 13S). If growth in *T. pyriformis* is reflective of growth in macrophages or pathogenicity in animals, the protozoan could serve as a model for intracellular growth. Further, it is tempting to speculate that there exists a relationship between the extent of intracellular growth of these mycobacteria in protozoa and amoebae and their ability to grow in animal macrophages and subsequent virulence. *M. scrofulaceum* is not as virulent as are *M. avium* and *M. intracellulare* for chickens (2, 25, 27, 33) and mice (33). Although caution must be taken in comparing representative strains of different species, the reduced pathogenicity of *M. scrofulaceum* may be reflected by its weak intracellular growth in *T. pyriformis*.

Based on these data and those of others (6, 22, 36) phagocytic protozoa are likely reservoirs for *M. avium*, *M. intracellulare*, and *M scrofulaceum* in the environment. The number of intracellular fluorescent cells of *T. pyriformis* infected with *M. avium* strain A5 remained at four per protozoan for up to 25 days. Water-grown cells of *M. avium* strain A5 were phagocytized and grew 24-fold over the course of the 5-day incubation. Because MAIS organisms grow in water (13), their persistence is not dependent upon protozoa. However, MAIS growth rates in water (13) are substantially lower than those in protozoa. Thus, the increase in MAIS numbers provided by intracellular parasitism of protozoa would be expected to significantly increase the number of these opportunistic pathogens in water. The fact that the number of *M. avium* cells per protozoan remained at the same value suggests that intracellular mycobacterial numbers are subject to regulation. That regulation may serve as a useful model for understanding persistent infections. Although the protozoa and infected cultures were incubated at 30°C to ensure reproducibility of the results, intracellular growth of MAIS in *T. pyriformis* was observed, albeit at a reduced rate, at room temperature (data not shown). Finally, *M. avium* survived encystment and germination of *T. pyriformis* (this study) and *A. polyphaga* (36). Because of the extreme resistance of these mycobacteria to chlorine-based disinfectants and ozone (37), survival of MAIS in protozoan cysts may not contribute significantly to survival following disinfection of drinking waters. Thus, the principal effect of intracellular parasitism of MAIS in protozoa may be the increase in population size and possible increases in virulence.

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