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Survival of Environmental Mycobacteria in *Acanthamoeba polyphaga*

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Free-living amoebae in water are hosts to many bacterial species living in such an environment. Such an association enables bacteria to select virulence factors and survive in adverse conditions. Waterborne mycobacteria (WBM) are important sources of community- and hospital-acquired outbreaks of nontuberculosis mycobacterial infections. However, the interactions between WBM and free-living amoebae in water have been demonstrated for only few *Mycobacterium* spp. We investigated the ability of a number ($n = 26$) of *Mycobacterium* spp. to survive in the trophozoites and cysts of *Acanthamoeba polyphaga*. All the species tested entered the trophozoites of *A. polyphaga* and survived at this location over a period of 5 days. Moreover, all *Mycobacterium* spp. survived inside cysts for a period of 15 days. Intracellular *Mycobacterium* spp. within amoeba cysts survived when exposed to free chlorine (15 mg/liter) for 24 h. These data document the interactions between free-living amoebae and the majority of waterborne *Mycobacterium* spp. Further studies are required to examine the effects of various germicidal agents on the survival of WBM in an aquatic environment.

Mycobacteria are a large group of microorganisms that inhabit a diverse range of natural environments. Environmental mycobacteria are a frequent cause of opportunistic infection in human beings and livestock (23, 56, 76). There is growing recognition in recent years that water is an important vehicle of transmission of environmental mycobacteria. This is based on the fact that in the recent past contaminated water supply systems have been responsible for several hospital and community outbreaks of mycobacterial infections (16, 77, 78, 79, 82, 83). These included infections as diverse as life-threatening pneumonia in patients with artificial ventilation, cystic fibrosis (54), and chronic granulomatous disease (79); outbreaks of skin infection following liposuction (51); furunculosis after domestic footbaths (77, 83); mastitis after body piercing (73); and abscess formation in intravenous drug users (26). In one instance (79, 82), workers exposed to contaminated industrial effluents developed pneumonia due to an environmental mycobacterium.

In hospital therapy pools, waterborne mycobacteria (WBM) may represent about 33% of the microorganisms present in water and about 80% of those in air in the vicinity of such a contaminated water source (5). WBM include both rapidly and slowly growing *Mycobacterium* spp., depending on whether they require a week or less for the production of visible colonies in solid medium. Examples of WBM include among other species the *Mycobacterium avium* complex, *Mycobacterium gordonae*, *Mycobacterium malmoense*, *Mycobacterium simiae*, *Mycobacterium marinum*, *Mycobacterium tusciae*, and *Mycobacterium lentiflavum*, which have been found in natural fresh waters (72, 76). *Mycobacterium mucogenicum*, *Mycobacterium kansasii*, *M. gordonae*, *Mycobacterium flavescens*, *Mycobacterium aurum*, *Mycobacterium fortuitum*, *Mycobacterium peregrinum*, and *Mycobacterium chelonae* have been isolated from public potable water (17, 47, 76). In water, *M. avium* and *Mycobacterium intracellulare* can survive, even under low oxygen tension (13, 41). Furthermore, *M. intracellulare* can remain viable for a year in deionized sterile water (6). Some WBM such as the *M. avium* complex, *Mycobacterium xenopi*, *Mycobacterium phlei*, and *M. chelonae* can withstand extreme temperatures and contaminate ice machine water (61). *M. fortuitum* and *M. chelonae* can form biofilms on Silastic rubber disks even under running water (34).

Free-living amoebae including *Acanthamoeba* spp. are commonly found in natural aquatic systems, water supplies, and cooling systems (37, 50), usually feeding on bacteria (68). It was shown that amoebae host several intracellular pathogens including *Legionella* spp., *Chlamydia* spp., *Parachlamydia* spp., *Listeria* spp., *Burkholderia* spp., *Campylobacter jejuni*, *Helicobacter pylori*, *Pasteurella multocida*, *Salmonella enterica*, *Francisella tularensis*, and *Simkania negevensis* (10, 32, 38). The association, mentioned above, of *Legionella pneumophila* with *Acanthamoeba* spp. may select organisms better capable of surviving in the hostile environment of mammalian phagocytic cells (35). This is supported by the fact that *Legionella* spp. use the same set of genes to establish themselves in *Acanthamoeba* spp. and in mammalian cells (27, 62).

The interactions between WBM and free-living amoebae are poorly understood. A number of *Mycobacterium* species, including *M. avium*, *M. marinum*, *M. simiae*, *M. phlei*, *Mycobacterium smegmatis*, and *M. fortuitum*, live intracellularly in amoebae (42). *M. avium* was shown to grow in *Acanthamoeba* spp. (15, 66). Recently, we used an amoebal coculture system for the isolation of *Mycobacterium massiliense* from clinical specimens (1).

In our present work, we compared levels of intra-amoebal penetration and intracystic survival of 26 environmental *Mycobacterium* spp. We also examined the effects of chlorination on the intra-amoebal growth and survival of these *Mycobacterium* species.

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TABLE 1. List of *Mycobacterium* sp. strains used in this study

Species	Strain	Source	Survival after infection of *A. polyphaga* in:		Reference
			Trophozoites	Cysts	
M. abscessus	CIP 104536^T	Abscess	+	+	44
M. chelonae	CIP 104535^T	Tortoise, tubercle	+	+	43
M. immunogenum	CIP 106684^T	Metal working fluid	+	+	82
M. mucogenicum	ATCC 49650^T	Thyroglossal duct cyst	+	+	64
M. fortuitum	CIP 104534^T	Abscess	+	+	20
M. peregrinum	CIP 105382^T	Bronchial aspiration	+	+	44
M. smegmatis	ATCC 19420^T	Genital secretions	+	+	48
M. goodii	ATCC 700504^T	Heel	+	+	14
M. mageritense	CIP 104973^T	Sputum	+	+	22
M. aurum	CIP 104465^T	Soil	+	+	74
M. kansasii	CIP 104589^T	Fatal infection	+	+	36
M. szulgai	CIP 104532^T	—[a]	+	+	49
M. malmoense	CIP 105775^T	Lung tissue	+	+	60
M. terrae	CIP 104321^T	Sputum, gastric lavage	+	+	80
M. marinum	CIP 104528^T	Saltwater fish	+	+	8
M. tusciae	CIP 106367^T	Cervical lymph node	+	+	71
M. gordonae	CIP 104529^T	Gastric lavage	+	+	12
M. intracellulare	CIP 104243^T	—	+	+	58
M. avium subsp. *avium*	CIP 104244^T	Diseased hen, liver	+	+	69
M. simiae	CIP 104531^T	Indian monkey	+	+	39
M. bohemicum	CIP 105811^T	Sputum	+	+	57
M. lentiflavum	CIP 105465^T	Spondylodiscitis	+	+	65
M. massiliense	CIP 108297^T	Bronchoalveolar fluid	+	+	1
M. septicum	ATCC 700731^T	Catheter tip of centrally located Hickman	+	+	59
M. porcinum	CIP 105392^T	Swine, lymph node	+	+	75
M. gastri	CIP 104530^T	Gastric lavage fluid	+	+	80

[a] —, source unknown.

MATERIALS AND METHODS

Mycobacterium strains. The type strains used in this study are listed in Table 1. The identification of species was establishedusing 16S rRNA and *rpoB* gene sequences. The primers used were Fd1 (5′-AGAGTTTGATCATGGCTCAG-3′) and Rp2 (5′-ACGGCTACCTTGTTACGACTT-3′) for 16S rRNA gene (81) and Myco-F (5′-GGCAAGGTCACCCCGAAGGG-3′) and Myco-R (5′-AGCG GCTGCTGGGTGATCATC-3′) for the *rpoB* gene (2). The strains had been held in skim milk and kept at −20°C before they were used. When required, each strain was cultured in Middelbrook 7H9 liquid medium. For subculture, we used Middelbrook and Cohn 7H10 agar (Becton Dickinson, Le Pont de Claix, France). All subcultures were done at 30°C. The minimum durations of incubation were 5 and 7 days for rapidly growing mycobacteria (RGM) and slowly growing mycobacteria (SGM), respectively.

Amoebal coculture and light-microscopic detection of *Acanthamoeba polyphaga* infected with mycobacteria. The strain (Linc-AP1) of *A. polyphaga* used was a donation by T. J. Rowbotham, Public Health Laboratory, Leeds, United Kingdom. It was grown at 28°C for 4 days in 150-cm^3 culture flasks (Corning, New York) containing 30 ml of peptone-yeast extract-glucose (PYG) broth (29, 30, 46). Amoebal cells were harvested when their average concentration reached a level of 5×10^5 cells/ml of broth. The harvested cells were then centrifuged at 2,000 rpm for 10 min, and the pellet was suspended twice in 30 ml of Page's modified Neff's amoeba saline (PAS) (29, 46). One milliliter of this suspension was dropped into each well of a 12-well microplate (Corning, New York) and incubated at 33°C for 7 days. The microplate, prepared as described above, was used for culturing mycobacteria. Each well of the microplate was inoculated with mycobacterium cells (concentration, 10^5 mycobacteria/ml) suspended in phosphate-buffered saline. The final concentration of mycobacterium cells in the well was 10^4 mycobacteria/ml. As a control, 1 μl of the suspension of mycobacterium cells (concentration, 10^4 mycobacteria/ml of PAS) was dropped into each well of a 12-well control microplate. The microplate was centrifuged at 4,000 rpm for 30 min and incubated at 33°C under an atmosphere humidified with 5% CO_2. After incubation for 48 h, the monolayer was washed three times with PAS, before being treated with amikacin (100 μg of amikacin for each milliliter of PAS) for 2 hours. This was done to kill any remaining extracellular or adherent mycobacteria (15, 67). Amikacin was removed from the monolayer by washing with PAS. After the washing, the monolayer was incubated in 1 ml of PAS for 3 days. In parallel, the supernatant obtained after washing was cultured in appropriate axenic medium. The microplate was examined daily to detect the presence of cytopathic effect. After gentle shaking and cytocentrifugation at 800 rpm for 10 min, mycobacteria were detected inside amoebal trophozoites as described by Gimenez (28), by Ziehl-Neelsen staining, and by Gram staining in amoebal cocultures that were 1 day, 3 days, and 5 days old, respectively. Intra-amoebal mycobacteria were released by lysing the monolayer with 1 ml of 0.5% sodium dodecyl sulfate, followed by two successive passages through a 27-gauge needle. The presence of viable mycobacteria was documented by detecting the presence of a colony of mycobacteria formed on Middlebrook 7H10 agar that had been inoculated with 200 μl of the cell lysate. All experiments were repeated three times.

Encystment. For encystment, we used amoebal cocultures that were 48 hours old. Ten milliliters of amoebal coculture (concentration, 5×10^5 amoebal cells/ml of PAS) was taken in 25-cm^3 culture flasks (Corning) and infected with 1 ml (concentration, 10^5 mycobacterium cells/ml of PAS) of mycobacterium suspension in PAS. The supernatant was discarded, and the amoebal monolayer was rinsed once with encystment buffer (0.1 M KCl, 0.02 M Tris, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$) before being incubated (at 33°C for 3 days) in fresh encystment buffer (66). The cysts formed were centrifuged at 4,000 rpm for 30 min. Then, they were washed three times with PAS by centrifugation. In order to kill extracellular mycobacteria, the pellet was resuspended in sodium hypochlorite (Javel oxena, Portes-Les-Valences, France) solution (concentration, 15 mg of chlorine/ml of the solution) and held at 33°C for 24 h. The concentration of chlorine we used was well in accord with that recommended for decontamination of water reservoirs throughout France. The cysts were washed thrice with PYG medium. One half of the sample so washed was processed for electron microscopy and the other half incubated (at 33°C for 7 days) in PYG medium. Viable cysts became trophozoites and eventually lysed due to the large number of intracellular mycobacteria. The process of excystment was verified by light-microscopic examination of Ziehl-Neelsen smears and by observing viable mycobacteria grown in Middlebrook 7H10 agar medium. In parallel with electron microscopy studies, cysts were prepared and stored at room temperature for 15 days. All experiments were repeated three times.

Ultrastructural study. To eliminate noningested mycobacteria, the amoebal monolayer inoculated with mycobacteria and amoebal cysts were washed twice

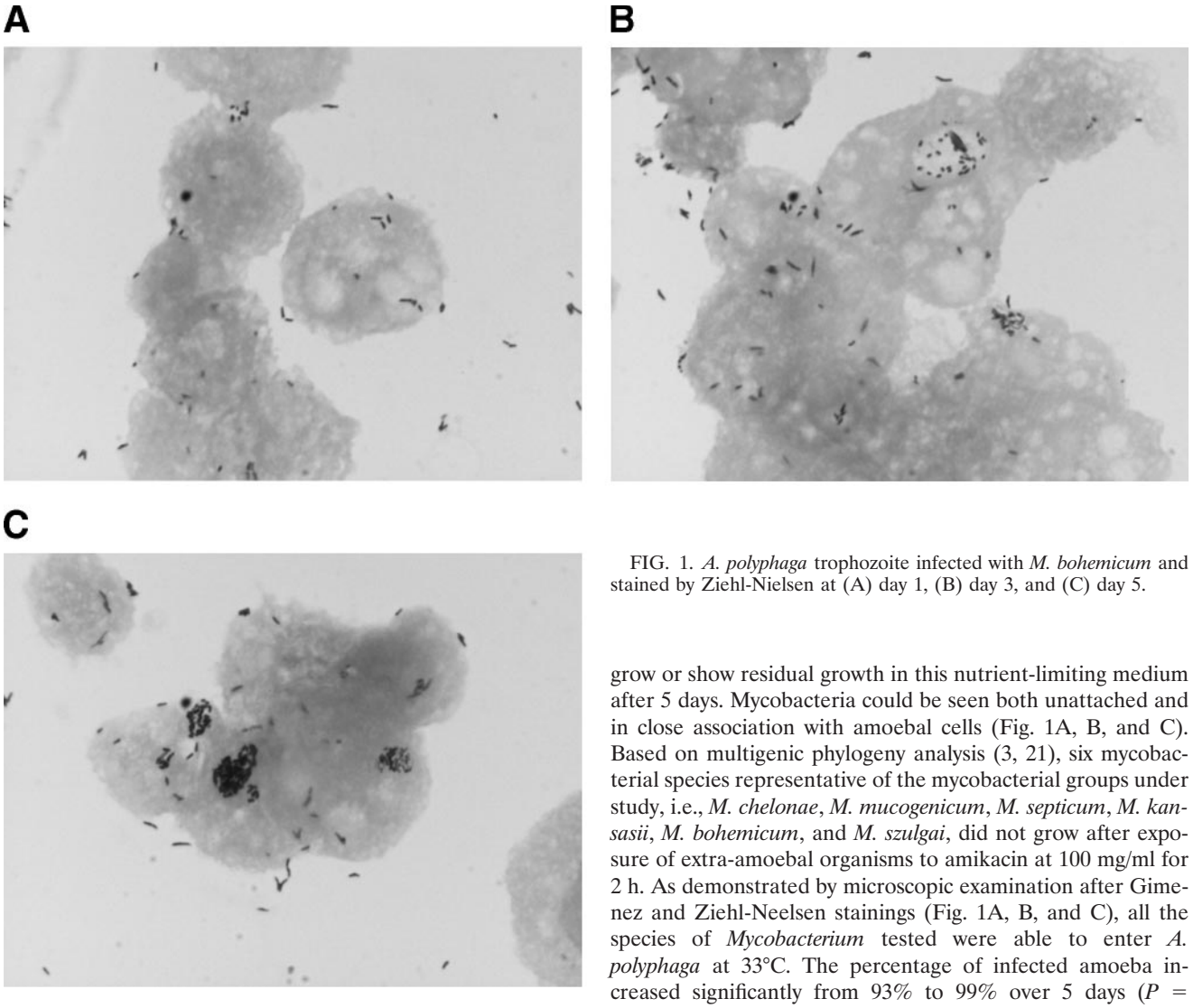

FIG. 1. *A. polyphaga* trophozoite infected with *M. bohemicum* and stained by Ziehl-Nielsen at (A) day 1, (B) day 3, and (C) day 5.

with sterile phosphate-buffered saline. They were then incubated overnight in monophosphate buffer (pH 12) before being fixed (1 h at 4°C) with 1% osmium tetroxide. The fixed samples were dehydrated by successive washing with graded (from 25 to 100%) concentrations of acetone. Then, the samples were successively incubated (for 1 h) in a 50% (vol/vol) acetone-Epon suspension and Epon (overnight) before being embedded in Araldite (Fluka, St Quentin Fallavier, France). Ultrathin sections were cut from the blocks using an ultracut microtome (Reichert-Leica, Marseille, France) before being deposited on copper grids coated with Formvar (Sigma-Aldrich, Taufkirchen, Germany). The ultrathin sections so mounted were stained for 10 min with methanol-uranyl acetate and lead nitrate with sodium citrate solution (30). The grids were examined under a transmission electron microscope (Morgani 268D; Philips, Eindhoven, The Netherlands).

Stastitical analysis. Repeated-measures analysis of variance was performed using the SAS v9.1.3 (SAS Institute Inc., Cary, NC) software to test whether the amoebal infection rates differed within and between mycobacterial types. Tests were two sided, and P values <0.05 were considered significant.

RESULTS

Survival of mycobacteria in *A. polyphaga*. All the species of *Mycobacterium* tested survived in PAS. However, they did not grow or show residual growth in this nutrient-limiting medium after 5 days. Mycobacteria could be seen both unattached and in close association with amoebal cells (Fig. 1A, B, and C). Based on multigenic phylogeny analysis (3, 21), six mycobacterial species representative of the mycobacterial groups under study, i.e., *M. chelonae*, *M. mucogenicum*, *M. septicum*, *M. kansasii*, *M. bohemicum*, and *M. szulgai*, did not grow after exposure of extra-amoebal organisms to amikacin at 100 mg/ml for 2 h. As demonstrated by microscopic examination after Gimenez and Ziehl-Neelsen stainings (Fig. 1A, B, and C), all the species of *Mycobacterium* tested were able to enter *A. polyphaga* at 33°C. The percentage of infected amoeba increased significantly from 93% to 99% over 5 days ($P = 0.0005$) (Table 2). This percentage was not significantly different between RGM and SGM ($P = 0.34$). Species were able to survive in the intracellular milieu of the trophozoites of *A. polyphaga* for the duration of the experiment (5 days). This was confirmed by positive subcultures although quantification of subcultured mycobacteria was not attempted. Mycobacterial species could be easily observed in the amoebal cytoplasm even on day 1 postinfection. The majority of RGM caused extensive vacuolization of trophozoites after 48 h of coculture. In contrast, it took at least 96 h for SGM to induce the formation of visible vacuoles inside amoeba. The amoeba cell infected as described above showed one or more than one vacuole containing mycobacteria (Fig. 1A, B, and C). The presence of mycobacteria inside the vacuole was confirmed by transmission electron microscopy (Fig. 2A, B, C, and D). The vacuoles containing mycobacteria were surrounded by a large number of host mitochondria (Fig. 2D). However, we did not observe any ultrastructural change in the mitochondria of the host. *A. polyphaga* released free mycobacteria (Fig. 3A and B) or 2.5- to 6.5-μm vesicles containing mycobacteria (Fig. 3C and D).

Mycobacteria inside *A. polyphaga* cysts. All the 26 *Mycobacterium* spp. tested survived inside *A. polyphaga* cysts for 15 days

TABLE 2. Percentages of *A. polyphaga* trophozoites infected with different species of nontuberculous mycobacteria over a 5-day coculture[a]

Age of *A. polyphaga* culture (days)	Mean %[b] (±SD) of *A. polyphaga* trophozoites infected by:			
	M. mucogenicum	*M. septicum*	*M. bohemicum*	*M. szulgai*
1	93.0 ± 3.0	93.0 ± 4.6	96.0 ± 3.6	97.6 ± 3.2
3	97.3 ± 1.5	97.3 ± 2.1	99.0 ± 1.0	98.3 ± 1.5
5	96.7 ± 3.2	98.3 ± 1.5	96.7 ± 1.5	99.7 ± 0.6

[a] The observed percentages were not significantly different.
[b] Percentage of *A. polyphaga* cells with at least one intravacuolar mycobacterium organism.

at room temperature. We examined the fate of mycobacteria inside *A. polyphaga* cysts by transmission electron microscopy. Figure 4A and B document one such cyst containing numerous *M. chelonae* and *M. abscessus* bacilli, respectively. They were visible in the spaces between the two walls (i.e., inner and outer walls) of the cyst. Some species (e.g., *M. septicum*) were observed to have been present on the inner side of the outer wall and in the cytoplasm of the cyst (Fig. 4C and D). Furthermore, *M. septicum* was present in dead cysts (data not shown). Less than 3% of infected cysts harbored bacilli in the cytoplasm, and <1% of infected cysts harbored bacilli in the outer wall.

The effect of chlorination on the viability of mycobacterium was examined. The mycobacterium species exposed, as described above, to free chlorine (15 mg/liter) for 24 h did not grow in Middelbrook 7H10 agar medium. In contrast, the concentration of chlorine we used was not destructive to *A. polyphaga* cysts, irrespective of whether they had been infected or were noninfected. This is evident by the fact that well over 50% of the cysts that were exposed to free chlorine for 24 h excysted in PYG medium. The abilities of amoeba cysts, both infected with mycobacteria and noninfected, to revert to trophozoites under optimal growth conditions were examined by microscopy. Samples were obtained from these cultures and subcultured in Middlebrook 7H10 agar. The results were positive for mycobacteria. The morphological features of the bacterial colonies grown in Middlebrook 7H10 agar were consistent with those of acid-fast bacilli.

DISCUSSION

Twenty-six *Mycobacterium* spp. tested survived within *A. polyphaga* trophozoites for 5 days (Table 1). We observed that the addition of amikacin to the incubation buffer after 2 hours of coculture killed extra-amoebal organisms due to the bactericidal effect of this antibiotic, as previously described (15, 67). No difference was observed in the numbers of intracellular mycobacteria in the presence or in the absence of amikacin (15). Previous studies have suggested an association between free-living amoeba and various *Mycobacterium* spp. in aquatic environments (23, 32, 56, 76). Recently, such an association has been documented in a hospital water supply system (68). Our data show, for example, that free-living amoeba may be part of the reservoir of *M. septicum*. This newly described mycobacterium was isolated from human samples (4, 59) and further from biofilms in a drinking water distribution system (63) and was recently associated with a devastating outbreak in a fish colony (40).

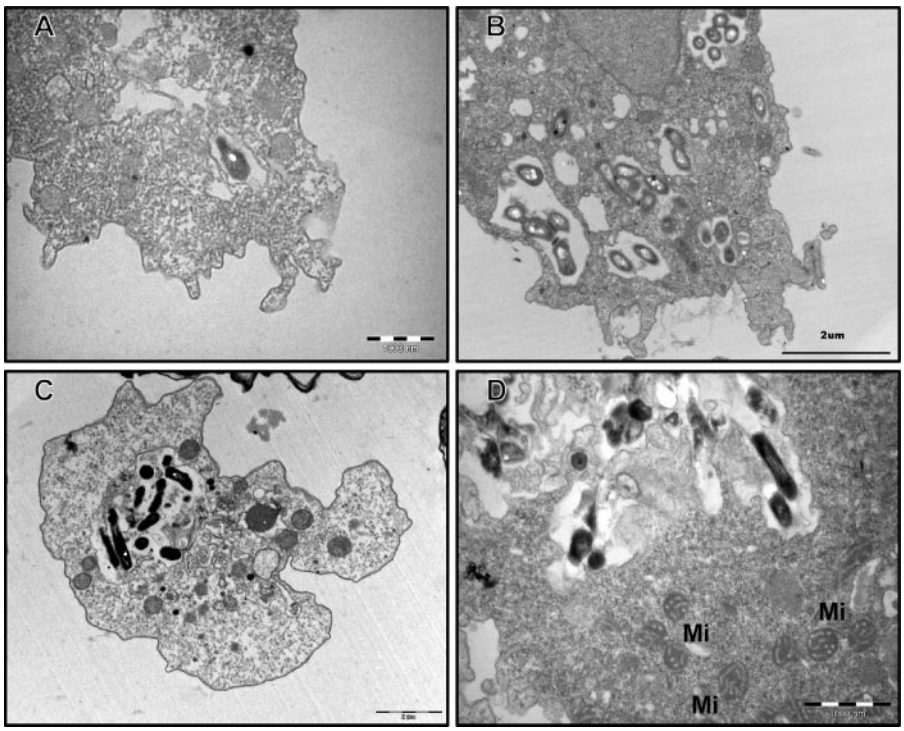

FIG. 2. Transmission electron-microscopic observation of *M. mucogenicum* (2-h coculture; A), *M. massiliense* (3-day coculture; B), *M. septicum* (5-day coculture; C), and *M. terrae* (5-day coculture; D) cocultivated with *A. polyphaga* trophozoites. The bacteria are seen inside an amoebal vacuole. Mi, mitochondria.

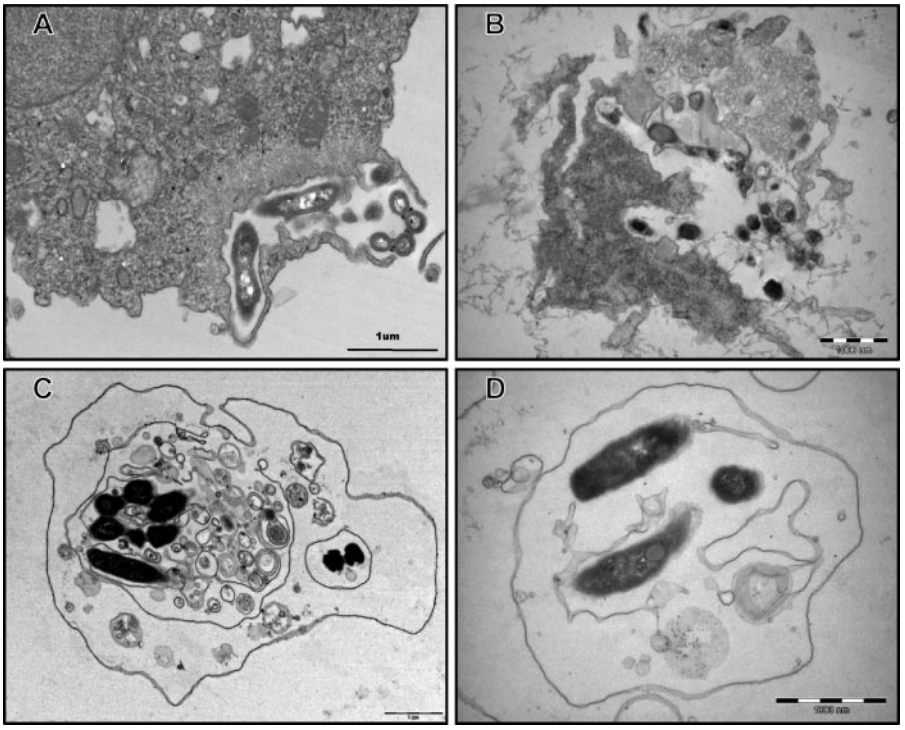

FIG. 3. Transmission electron-microscopic observation of *A. polyphaga* relapsing free *M. massiliense* (A), relapsing *M. terrae* through lysis (B), and relapsing vesicles containing *M. septicum* (C and D).

Furthermore, these species survived within the cysts of *A. polyphaga* for 15 days. The possibility that the cysts of *A. polyphaga* might have been contaminated by mycobacteria from an external source was considered extremely unlikely. To avoid contamination, we carefully and repeatedly washed the amoebal monolayer. For each washing step, we used fresh encystment buffer. We also exposed the cysts to chlorination (15 mg/liter) for 24 h. We have herein demonstrated that this

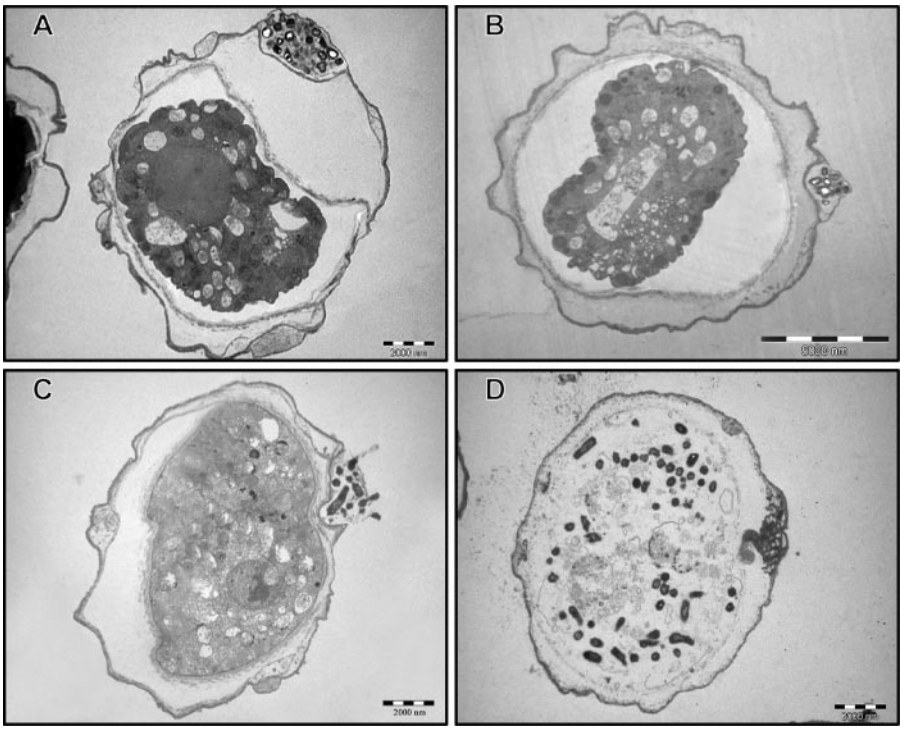

FIG. 4. Transmission electron-microscopic observation of *A. polyphaga* cysts containing *M. chelonae* (A) and *M. abscessus* (B) within a double cell wall and *M. septicum* within a double cell wall (C) and in the cytoplasm (D).

level and duration of exposure to free chlorine was germicidal to the *Mycobacterium* spp. tested. The cysts are double-walled, resilient entities which survive exposure to temperature between −20°C and +42°C (7). We found that *M. chelonae* and *M. abscessus* survive within the amoeba cyst. This fact could explain why these species, which cannot grow at 42°C as free organisms, were recovered in large numbers from a hot-water drinking water distribution system (25). Also, *M. chelonae* can withstand extreme temperatures and can contaminate ice machine water (61).

The cysts of *A. polyphaga* can withstand germicidal compounds commonly used for decontaminating bronchoscopes (31). This means that the cysts of *A. polyphaga* may remain viable in bronchoscopes even after they have been decontaminated with germicides. In fact, contaminated bronchoscopes have been the sources of infections due to some nontuberculosis mycobacterial species, including *M. chelonae* and *M. kansasii*, in immunocompromised patients (18, 55, 78, 84). Likewise, contamination of hospital equipment and medication was traced to the ubiquitous presence of these organisms in tap water (70, 78). Their resistance to commonly used disinfectants was responsible for pseudo-outbreaks of infections associated with surgical implants, health care-associated septicemia, and lung disease following bronchoscopy (9, 24, 45, 78). Our data also indicate that mycobacterium-infected cysts can withstand a chlorine concentration of 15 mg/liter whereas the trophozoites of *A. polyphaga* are sensitive to chlorine at a concentration of 1.25 mg/liter (19). Indeed, we recovered viable mycobacteria from the cysts of *A. polyphaga* that had been exposed to such a chlorine concentration. This is a source of concern. These data suggest that encystment of mycobacteria may be one of the many mechanisms used by these organisms to resist the germicidal effect of chlorine in water distribution systems. These data may also explain the differences in the mycobacterium populations observed in treated and untreated waters (72). In one pilot study, treatment of a water system with ozone or chlorine resulted in a dramatic shift in the bacterial population to the *Actinomyces* family, which includes mycobacterial species (53).

Previous studies have also documented the presence and growth of *M. avium* inside the trophozoites of *Acanthamoeba castellanii* (15). The growth in trophozoites enhanced the entry of this mycobacterium into amoebae, the intestinal epithelial cell line (HT-29), and macrophages (15). It also enhanced the abilities of *M. avium* to colonize the intestine and replicate in the livers and spleens of mice in a murine model of *M. avium* infection (15). Furthermore, it was found that mycobacteria cultured into amoebas were better able to survive within macrophages and that interaction of *M. avium* with environmental amoebae enhances virulence (15). Our findings are in agreement with the speculation that bacteria resistant to amoebae may be resistant to macrophages and vice versa and therefore that amoebae select pathogenic microorganisms (33). Compared to those living within macrophage-like cell lines (e.g., U937), *M. avium* living inside the trophozoites of *A. castellanii* were shown to be more resistant to rifabutin, azithromycin, and clarithromycin (52). Our data now extend previous data to *M. intracellulare*, a species closely related to *M. avium*. We have also demonstrated the survival of *M. smegmatis* inside the trophozoites of *A. polyphaga*. This contradicts the findings of others (15), while *M. smegmatis* was shown to have persisted inside HEp-2 epithelial cells (11).

In conclusion, 26 environmental *Mycobacterium* spp. survived within amoebal trophozoites and cysts for various lengths of time. This fact may have implications for the mode of transmission of these microorganisms. It has been shown previously that the association between amoeba and *Legionella pneumophila* in an aquatic environment enabled this bacterium to select virulence factors and survive in such a hostile environment (27). This may also be true for environmental *Mycobacterium* spp. The environmental *Mycobacterium* spp. living within the cysts of *A. polyphaga* can withstand the concentration of free chlorine normally used for the treatment of water in municipal water supply systems. We believe that testing encysted mycobacteria may be necessary to properly evaluate decontamination procedures for water and endoscopes.

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