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Mycobacterium gilvum Illustrates Size-Correlated Relationships between Mycobacteria and *Acanthamoeba polyphaga*

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Mycobacteria are isolated from soil and water environments, where free-living amoebae live. Free-living amoebae are bactericidal, yet some rapidly growing mycobacteria are amoeba-resistant organisms that survive in the amoebal trophozoites and cysts. Such a capacity has not been studied for the environmental rapidly growing organism *Mycobacterium gilvum*. We investigated the ability of *M. gilvum* to survive in the trophozoites of *Acanthamoeba polyphaga* strain Linc-AP1 by using optical and electron microscopy and culture-based microbial enumerations in the presence of negative controls. We observed that 29% of *A. polyphaga* cells were infected by *M. gilvum* mycobacteria by 6 h postinfection. Surviving *M. gilvum* mycobacteria did not multiply and did not kill the amoebal trophozoites during a 5-day coculture. Extensive electron microscopy observations indicated that *M. gilvum* measured 1.4 ± 0.5 μm and failed to find *M. gilvum* organisms in the amoebal cysts. Further experimental study of two other rapidly growing mycobacteria, *Mycobacterium rhodesiae* and *Mycobacterium thermoresistibile*, indicated that both measured <2 μm and exhibited the same amoeba-mycobacterium relationships as *M. gilvum*. In general, we observed that mycobacteria measuring <2 μm do not significantly grow within and do not kill amoebal trophozoites, in contrast to mycobacteria measuring >2 μm ($P < 0.05$). The mechanisms underlying such an observation remain to be determined.

Nontuberculous mycobacteria are environmental organisms (1, 2) found in soil (3), the marine environment (4), and fresh water (5, 6). They are recovered from water samples also colonized by free-living amoebae (FLA) (7–9). Despite the fact that FLA are bactericidal, several nontuberculous mycobacteria were found to be amoeba resistant, surviving within FLA trophozoites and cysts (10, 11). The latter act as Trojan horses protecting environmental mycobacteria from unfavorable conditions (7, 12, 13).

Amoeba-resistant mycobacteria include slowly growing mycobacteria (SGM), such as *Mycobacterium avium* (14) and *Mycobacterium tuberculosis* (13, 15) complex mycobacteria and more than 25 different species of rapidly growing mycobacteria (RGM) (7, 11). The outcomes for such rapidly growing, amoeba-resistant mycobacteria depend on the mycobacterial species: some *Mycobacterium* species, such as *Mycobacterium septicum*, survive without multiplication into trophozoites (7), while other species, such as *Mycobacterium smegmatis* and *Mycobacterium chelonae*, multiply within the trophozoite (11, 16). Also, some mycobacteria, such as *Mycobacterium canettii*, escape the FLA before encystment (13), whereas the majority of *Mycobacterium* species survive within the amoeba cysts (13, 14).

Mycobacterium gilvum (formerly *Mycobacterium flavescens*) is an environmental mycobacterium isolated from river sediments on the basis of its ability to degrade polycyclic aromatic hydrocarbons, such as pyrene, as a sole source of carbon and energy (17, 18). It is able to form biofilm, and it is resistant to ampicillin but is susceptible to other antibiotics, including isoniazid (19). *M. gilvum* has rarely been isolated as an opportunistic pathogen (19), and no study regarding *M. gilvum*-amoeba relationships has yet been performed.

We therefore studied the relationships between *M. gilvum* and the trophozoites and cysts of the FLA *Acanthamoeba polyphaga* and derived features characterizing amoeba-mycobacterium relationships. To validate our observations with *M. gilvum*, we further studied two other rapidly growing mycobacteria, *Mycobacterium rhodesiae* and *Mycobacterium thermoresistibile*.

MATERIALS AND METHODS

***Mycobacterium* and *A. polyphaga* strains.** Strains *Mycobacterium senegalense* DSM-43656^T, *Mycobacterium conceptionense* DSM-45102^T, *Mycobacterium rhodesiae* DSM-44223^T, *Mycobacterium thermoresistibile* DSM-44167^T, *Mycobacterium chelonae* DSM-43804^T, *Mycobacterium smegmatis* DSM-43756^T, *Mycobacterium abscessus* DSM-44196^T, *Mycobacterium fortuitum* subsp. *fortuitum* DSM-46621^T, and *M. gilvum* DSM-45363^T were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). Mycobacteria were cultured in Middlebrook 7H9 liquid medium (Sigma-Aldrich, Lyon, France) and subcultured at 37°C on Middlebrook and Cohn 7H10 agar (Becton, Dickinson, Le Pont de Claix, France) for 3 days. The *A. polyphaga* Link-AP1 trophozoite strain (20) was cultured in peptone-yeast extract-glucose (PYG) medium at 32°C for 3 day as described previously (11, 13). In brief, *A. polyphaga* amoebae were suspended twice in Page's modified Neff's amoeba saline (PAS) to obtain 5×10^5 cells/ml, and 10 ml of this suspension was placed into 50-ml Falcon tubes (Becton, Dickinson, Le Pont de Claix, France).

Mycobacterium-amoeba coculture. Liquid cultures of the RGM *M. gilvum*, *M. rhodesiae*, and *M. thermoresistibile* were washed two times with sterile phosphate-buffered saline (PBS), and the pellet was suspended in PAS. Each 10 ml of the amoebal culture was inoculated with 1 ml of a suspension of 5×10^7 RGM/ml (multiplicity of infection, 1:10). As a control, *A. polyphaga*, *M. gilvum*, *M. rhodesiae*, and *M. thermoresistibile*

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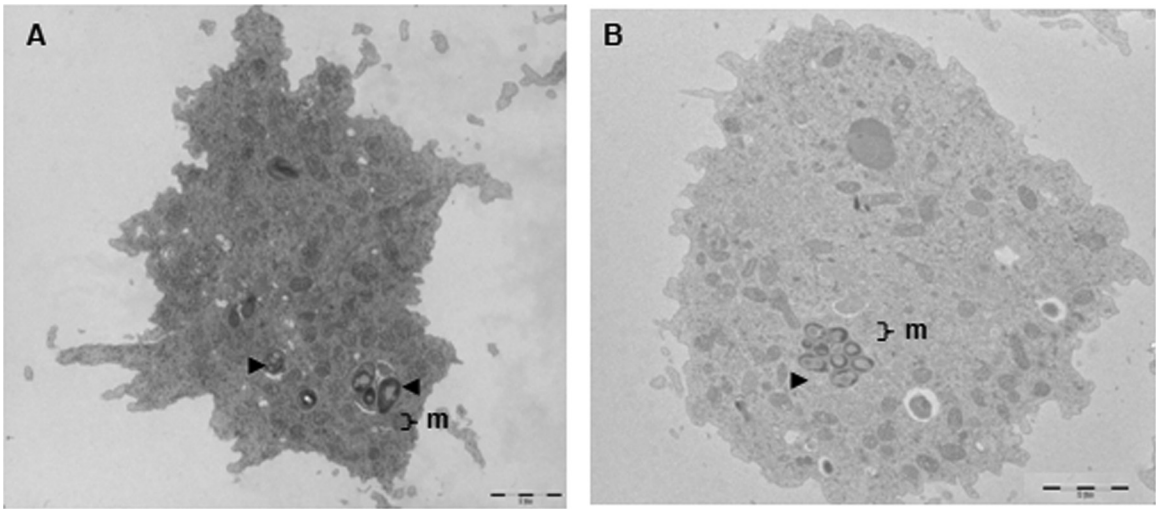

FIG 1 *M. gilvum* mycobacteria are internalized into amoebae. Transmission electron microscopy observation of *M. gilvum* (▶) cocultivated with *A. polyphaga* trophozoites at 0 h (A) and 72 h (B). m, mitochondria. Bars, 2 μm.

were cultured separately in PAS medium. After a 6-h incubation at 32°C, the coculture was washed two times with PAS to remove any remaining extracellular or adherent mycobacteria (11). After washing, the coculture was incubated in 10 ml of PAS for 5 days at 32°C.

The presence of intra-amoebal mycobacteria was determined by shaking the coculture, a 10-min centrifugation at 100 × *g*, and observation using a light microscope after Ziehl-Neelsen staining. In addition, the presence of viable mycobacteria inside amoebal trophozoites was assessed as previously described (11). In brief, at times of 0, 24, 48, 72, 96, and 120 h postinoculation, the *A. polyphaga* monolayer was lysed with 0.1% sodium dodecyl sulfate (Sigma-Aldrich) for 30 min and passed through a 26-gauge needle to ensure complete lysis of the amoebae. A 100-μl volume of lysate was plated onto 7H10 agar and incubated for 4 days at 37°C to determine the number of colonies (number of CFU) of intracellular mycobacteria. The viability of amoebae with and without bacteria was done using Trypan blue (0.4%; Sigma-Aldrich, Taufkirchen, Germany) and counting in a Glasstic slide chamber (HycoR, Garden Grove, CA). Experiments were done in triplicate. Negative controls remained negative in each experimental step.

Encystment of *M. gilvum*-infected amoebae. Amoebae were cultured with encystment buffer as described previously (11, 13). In brief, 10 ml of amoebal coculture (5×10^5 cells/ml of PAS) was infected with 1 ml (5×10^7 mycobacteria/ml of PAS) of *Mycobacterium* suspension in PAS for 6 h. The supernatant was discarded, and the amoebal monolayer was rinsed twice with encystment buffer before being incubated at 32°C for 3 days in fresh encystment buffer (11). Moreover, cysts corresponding to time zero were centrifuged at 1,000 × *g* for 10 min and washed three times with PAS before electron microscopy observation. Experiments were done in triplicate.

Ultrastructural studies. Ultrastructural observations were done as previously described (11). In brief, amoeba monolayers previously infected by *M. gilvum* and amoebal cysts were washed three times with sterile PAS to eliminate noningested mycobacteria and fixed (11). Then, the samples were successively incubated for 45 min in a 3:1, 2:2, or 1:3 (vol/vol) ethanol-Epon suspension and then in 100% Epon overnight with continuous shaking, before being embedded in an Epon 812 resin (Fluka, St. Quentin Fallavier, France) and incubated for 3 days at 60°C. Ultrathin sections (70 nm) were cut from the blocks using an ultracut microtome (Reichert-Leica, Marseille, France), before being deposited on Formvar-coated copper grids (Sigma-Aldrich). Ultrathin sections were stained for 10 min with 5% uranyl acetate and lead citrate, before being examined using a transmission electron microscope (Morgani 268D; Philips, Eindhoven, the Netherlands).

Mycobacterial size was determined after a 2-day culture in Middlebrook 7H9 medium at 37°C and after a 2-day preculture in Middlebrook 7H9 medium, followed by a 2-day culture in PAS medium at 37°C. The

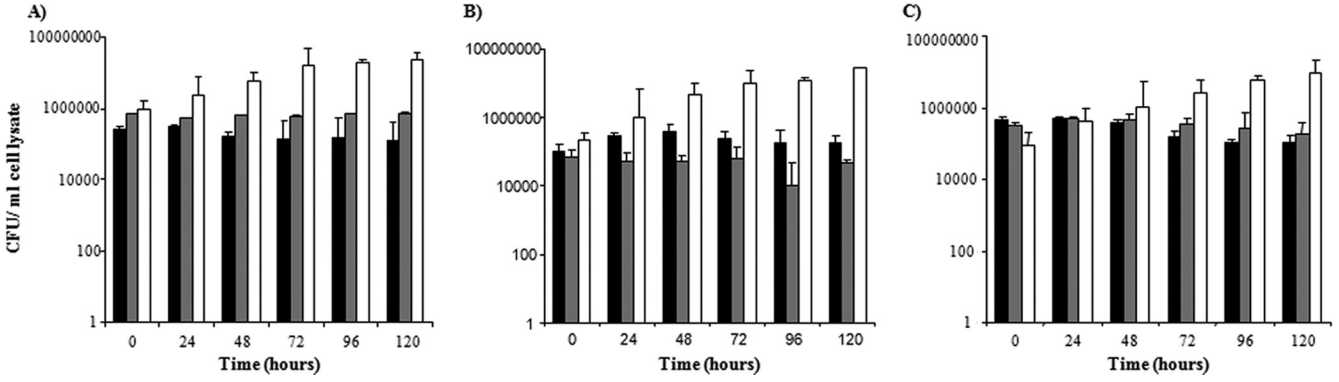

FIG 2 Growth of RGM within *A. polyphaga* trophozoites. *M. gilvum* (A), *M. rhodesiae* (B), and *M. thermoresistibile* (C) were cocultured with the free-living amoeba *A. polyphaga* (black bars), cultivated in PAS medium (gray bars), and cultivated in 7H9 complete medium (white bars). Each bar represents the mean of triplicate experiments. Standard errors are represented by error bars.

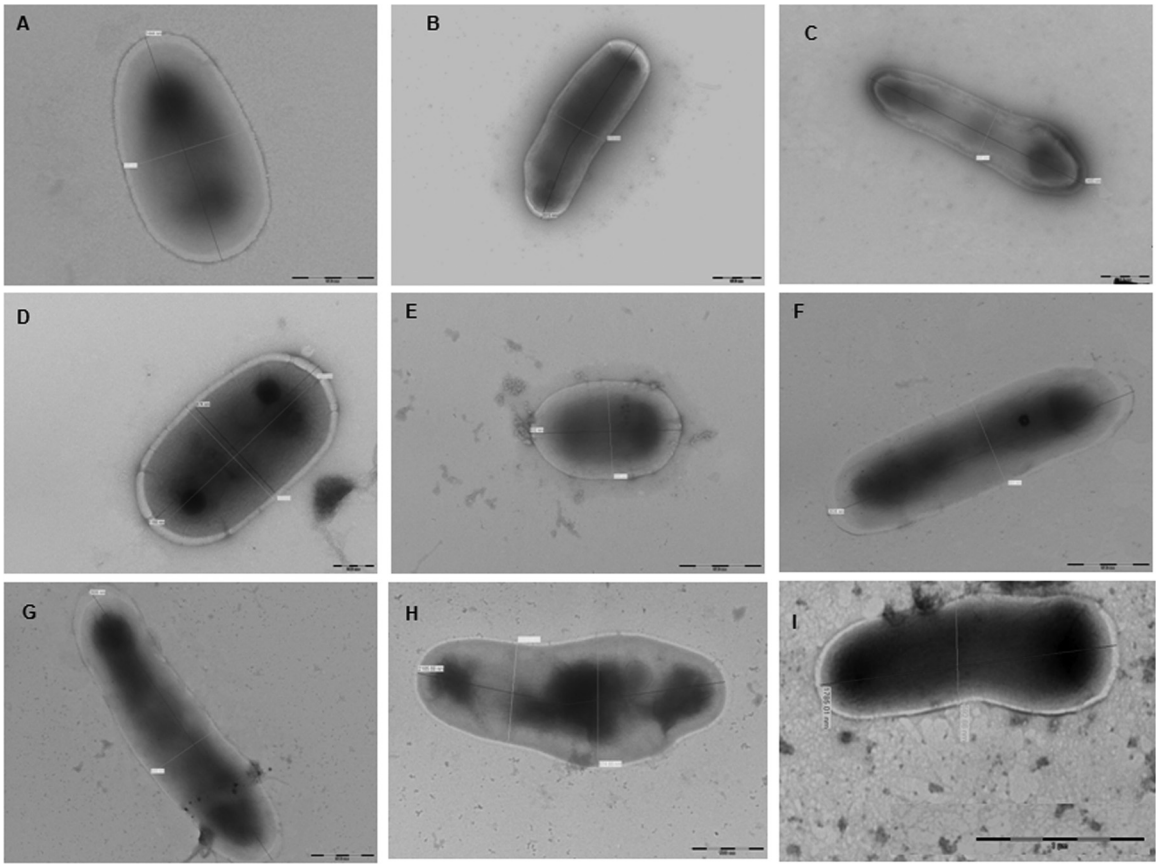

FIG 3 Mycobacterial size in 7H9 medium. The sizes of *M. gilvum* (A), *M. senegalense* (B), *M. conceptionense* (C), *M. rhodesiae* (D), *M. thermoresistibile* (E), *M. chelonae* (F), *M. smegmatis* (G), *M. abscessus* (H), and *M. fortuitum* subsp. *fortuitum* (I) were determined by electron microscopy. The sizes were measured under the same cultures conditions for all mycobacteria (see Materials and Methods). Bars, 500 nm (A to C and E to H), 300 nm (D), and 1 μm (I).

size of the mycobacteria was measured by electron microscopic observation of 50 single mycobacteria to determine the median and standard deviation of the cell length.

Statistical analyses. All statistical analyses mentioned in this study were performed using the χ^2 test with a significance level of P equal to 0.05.

RESULTS

Survival of mycobacteria in *A. polyphaga* trophozoites and cysts. The number of noninfected (negative-control) and infected *A. polyphaga* trophozoites with RGM incubated in PAS at 32°C did not change significantly over the time of the experiment. After 6 h of coculture, 29% of *A. polyphaga* cells were found to be infected by *M. gilvum* mycobacteria, as confirmed by Ziehl-Neelsen staining. The number of *M. gilvum* organisms per trophozoite varied from 1 to 46 (mean, 17 ± 14 mycobacteria/trophozoite). Electron microscopy revealed mycobacteria in vacuoles surrounded by several mitochondria (Fig. 1A). We observed that the three tested RGM species survived but did not multiply over the 5-day coculture with amoeba at 32°C (Fig. 2). No significant difference in the number of mycobacteria with time was observed ($P = 0.1$). Mycobacteria survived in PAS, yet the numbers of *M. gilvum*, *M. rhodesiae*, and *M. thermoresistibile* CFU did not increase from day 0 to day 5 (negative control) (Fig. 2). Electron microscopy revealed precysts and mature cysts after 3 days of coculture. Careful electron microscopy observation of 300 cysts formed at that time failed to reveal any *M. gilvum* organism in *A. polyphaga* cysts (see Fig. S1 in the supplemental material).

RGM size measurement. When it was not available in literature, we measured the length of RGM cells under two different culture conditions (see Materials and Methods) using electron microscopy observation. In this study, after culture in 7H9 medium, the lengths measured were 2.1 ± 0.7 μm for *M. abscessus*, 2.3 ± 0.3 for *M. chelonae*, 2.6 ± 0.1 for *M. smegmatis*, 2.5 ± 0.5 μm for *M. conceptionense*, 1.7 ± 0.1 μm for *M. fortuitum* subsp. *fortuitum*, 1.9 ± 0.2 μm for *M. senegalense*, 1.3 ± 0.4 μm for *M. rhodesiae*, 1.1 ± 0.3 μm for *M. thermoresistibile*, and 1.4 ± 0.5 μm for *M. gilvum* (Fig. 3). After culture in 7H9 medium, the lengths measured were 2.2 ± 0.5 μm for *M. abscessus*, 2.1 ± 0.5 μm for *M. chelonae*, 2.8 ± 0.9 μm for *M. smegmatis*, 2.3 ± 0.9 μm for *M. conceptionense*, 1.8 ± 0.4 μm for *M. fortuitum* subsp. *fortuitum*, 1.8 ± 0.5 μm for *M. senegalense*, 1.6 ± 0.3 μm for *M. rhodesiae*, 1.4 ± 0.3 μm for *M. thermoresistibile*, and 1.3 ± 0.2 μm for *M. gilvum* (see Fig. S2 in the supplemental material). We observed that whatever the medium (7H9 or PAS), there was no significant difference in size between all studied mycobacterial strains ($P > 0.05$). The size of mycobacteria significantly correlated with intra-amoebal growth and amoeba killing, with mycobacteria measuring <2 μm not growing within and not killing amoebal trophozoites and with mycobacteria measuring >2 μm growing within and killing amoebal trophozoites ($P < 0.05$) (Table 1).

TABLE 1 Mycobacterium-amoeba relationships

Mycobacterial growth	Mycobacterium	Length[a] (μm)	Amoeba killer	Multiplies in trophozoites	Reference(s) or source
Rapid	*M. abscessus*	>2	+	+	16, 21; this study
	M. chelonae	>2	+	+	16, 22, 23; this study
	M. smegmatis	>2	+	+	11; this study
	M. conceptionense	>2	NS[b]	NS	This study
	M. fortuitum subsp. *fortuitum*	<2	NS	NS	7; this study
	M. senegalense	<2	NS	NS	This study
	M. rhodesiae	<2	−	−	This study
	M. thermoresistibile	<2	−	−	This study
	M. gilvum	<2	−	−	This study
Slow	*M. bovis*	<2	−	−	13
	M. leprae	<2	−	−	24
	M. avium	<2	−	−	7, 13, 14
	M. tuberculosis	<2	−	−	13
	M. canettii	>2	−	−	13

[a] In PAS or 7H9 medium.
[b] NS, not studied.

DISCUSSION

A. polyphaga and *Acanthamoeba castellanii* are two FLA routinely used to probe mycobacteria-FLA interactions, and *A. polyphaga* was used in this study (25–27). We observed that the rapidly growing mycobacteria *M. gilvum*, *M. rhodesiae*, and *M. thermoresistibile* penetrated into *A. polyphaga* trophozoites, a reproducible result obtained by using a low (1:10) multiplicity of infection. Previous studies have shown that the majority of RGM penetrated into amoebal trophozoites (7, 11), but our observation that *M. gilvum*, *M. rhodesiae*, and *M. thermoresistibile* could also be ingested by amoebal trophozoites has not been previously reported. We further observed that such intra-amoebal mycobacteria survived in the *A. polyphaga* trophozoites, a fact documented by both microscopic observations and microbial enumerations. This observation agrees with previous demonstrations of the intra-amoebal survival of *Mycobacterium septicum*, *Mycobacterium abscessus* (7), and *M. smegmatis* in *A. castellanii* (28, 29) and *A. polyphaga* (11). Furthermore, *M. gilvum* mycobacteria were observed in vacuoles, as previously observed for other RGM, such as *M. septicum*, *Mycobacterium mucogenicum*, *Mycobacterium massiliense*, and *M. smegmatis* in *A. polyphaga* (7, 11).

M. gilvum, *M. rhodesiae*, and *M. thermoresistibile* mycobacteria did not multiply within amoebae and did not kill the amoeba during the time of the experiment (5-day coculture). This is contrary to other RGM, such as *M. abscessus*, *M. chelonae*, *M. smegmatis*, *Mycobacterium monacense*, and *Mycobacterium neoaurum*, all of which multiply within trophozoites and kill the amoeba after a 5-day coculture (11, 16). These data indicate that not all the RGM are amoebal killers, suggesting that factors other than rapid growth may be involved in the mycobacterium-amoeba interac-

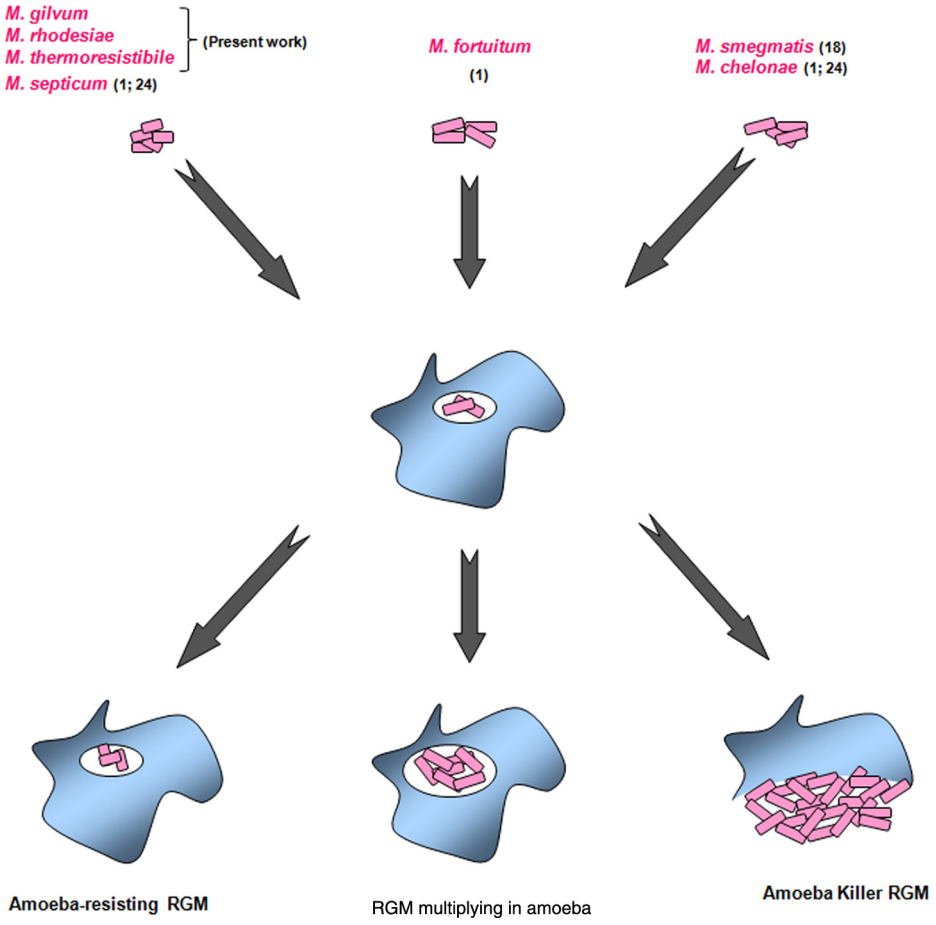

FIG 4 Spectrum of RGM-amoeba interactions.

tions. Accordingly, we observed here that intra-amoebal multiplication and amoeba killing significantly correlated with the size of the mycobacteria. Indeed, among mycobacteria belonging to the same species, organisms exhibiting a >2-μm size behave differently from the ones exhibiting a <2-μm size; larger RGM species measuring more than 2 μm penetrate, multiply, and kill the amoeba, contrary to the smaller RGM, such as *M. gilvum*, *M. rhodesiae*, and *M. thermoresistibile*, measuring less than 2 μm, which do not kill the amoeba (this study). This puzzling observation could be extended to SGM. We observed that the majority of studied SGM measuring less than 2 μm did not kill the amoeba; the notable exception was *M. canettii*, a species measuring more than 2 μm (13) which does not kill but instead escapes out of the amoeba.

Overall, our data indicate a significant correlation between the median size of mycobacteria and the outcome for mycobacteria in amoebae. This observation warrants further investigations to understand whether the size of the mycobacterium triggers the intra-amoebal outcome by itself or whether size is just a proxy for a biological property of mycobacteria, which has not been studied. Data presented herein suggest that the replication rate is not the biological factor. Also, we observed no correlation between the genome size of the studied mycobacteria and intra-amoebal survival (data not shown), pending additional genomic studies.

Extensive electron microscope observation failed to reveal any *M. gilvum* organisms in cysts. This observation extends previous observations made for other RGM, such as *M. smegmatis* (11). Combining morphological and cultural data indicates that the majority of RGM bypass the amoebal cyst after they are phagocytosed into the amoebal trophozoites. These data agree with the previous observations that all *M. canettii* organisms and the majority of *M. tuberculosis* organisms and nontuberculous organisms such as *M. smegmatis* (11) escape from the *A. polyphaga* precyst before its maturation, contrary to findings for *M. avium* organisms (14).

In conclusion, the characteristics of RGM-amoeba interactions may be wider than previously reported. The interactions may partly rely on the size of the RGM species and comprise the following: (i) RGM species smaller than 2 μm survive in amoebal trophozoites but not in the cysts and include *M. septicum* (7) and *M. gilvum* (present work); (ii) RGM longer than 2 μm survive in trophozoites and cysts and include *M. fortuitum* and *M. abscessus* (7, 16, 21); and (iii) RGM longer than 2 μm penetrate, multiply, and kill the amoeba and include *M. chelonae* (16) and *M. smegmatis* (11) (Fig. 4).

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We declare that we have no competing interests.

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