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Free-living amoebae, a training field for macrophage resistance of mycobacteria

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Abstract

Mycobacterium species evolved from an environmental recent common ancestor by reductive evolution and lateral gene transfer. Strategies selected through evolution and developed by mycobacteria resulted in resistance to predation by environmental unicellular protists, including free-living amoebae. Indeed, mycobacteria are isolated from the same soil and water environments as are amoebae, and experimental models using *Acanthamoeba* spp. and *Dictyostelium discoideum* were exploited to analyse the mechanisms for intracellular survival. Most of these mechanisms have been further reproduced in macrophages for mycobacteria regarded as opportunistic and obligate pathogens. Amoebal cysts may protect intracellular mycobacteria against adverse conditions and may act as a vector for mycobacteria. The latter hypothesis warrants further environmental and clinical studies to better assess the role of free-living amoebae in the epidemiology of infections caused by mycobacteria.

Keywords: *Acanthamoebae,* free-living amoebae (FLA), interaction, *Mycobacterium*, review
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Mycobacteria Evolved from an Environmental Ancestor

Mycobacteria are mycolic acid-containing, high guanine-cytosine-content bacterial organisms belonging to the *Actinobacteria* phylum [1]. Comparative genomics indicated that the most recent common ancestor of *Mycobacteria* was an environmental species [2,3] which evolved towards soil-borne mycobacteria, waterborne mycobacteria such as *Mycobacterium avium*, *Mycobacterium intracellulare,* and host-associated species including *M. avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* [4], *Mycobacterium tuberculosis* complex (MTC) species, and *Mycobacterium leprae*. Some species retained genes for dual lifestyle as in the case of *Mycobacterium marinum* [3]. In the latter species, genome synteny and size are more related to the saprophyte *Mycobacterium smegmatis* than to *M. tuberculosis*, despite the fact that *M. marinum* and *M. tuberculosis* share 80% of coding sequences [3]. This evolution mainly relied upon irreversible deletions of genomic fragments, illustrated by downsizing of the genome from environmental, generalist mycobacteria (*Mycobacterium vanbaalenii* genome, 6.49 Mb) towards host-associated, specialized species (*M. leprae* genome, 3.26 Mb) (Table 1). Current genomic data suggest that reductive evolution occurred independently in several *Mycobacterium* phyla [3]. Lateral gene transfer has also been demonstrated in some *Mycobacterium* species such as the *M. tuberculosis* complex [5] and *Mycobacterium abscessus* [6]. Transfer of the IS*6110* insertion sequence between *Mycobacterium smegmatis* and the *M. tuberculosis* complex species, and its discovery in another environmental *Mycobacterium* sp. strain JLS, suggested that these species once co-occupied a single environmental niche [7]. Reductive evolution of specialized mycobacteria is illustrated by the agent of Buruli ulcer, *Mycobacterium ulcerans*, which derived from an ancestor in common with *M. marinum* after genome size reduction from 6.63 to 5.63 Mb [8] and the acquisition of a 174-kb plasmid encoding a polyketide toxin mycolactone [9]. Of 140 species currently described in this genus, 133 (95%) are environmental organisms found in soil [10], air [11], and water [12–14], whereas seven are host-associated organisms (http://www.bacterio.cict.fr/m/mycobacterium.html).

TABLE 1. Comparative genome evolution of *Mycobacterium* sp.

Mycobacterium genomes (n = 18)	Access number	Length	GC content (%)	Intra-amibienne
M. smegmatis str. MC2 155	NC_008596	6 988 209 nt	67	+
M. marinum	NC_010612	6 636 827 nt	65	+
M. vanbaalenii PYR-1	NC_008726	6 491 865 nt	67	?
Mycobacterium sp. JLS	NC_009077	6 048 425 nt	68	?
Mycobacterium sp. KMS	NC_008705	5 737 227 nt	68	?
Mycobacterium sp. MCS	NC_008146	5 705 448 nt	68	?
M. ulcerans Agy99	NC_008611	5 631 606 nt	65	+
M. gilvum PYR-GCK	NC_009338	5 619 607 nt	67	?
M. avium 104	NC_008595	5 475 491 nt	68	++
M. abscessus	NC_010397	5 067 172 nt	64	?
M. avium subsp. paratuberculosis K-10	NC_002944	4 829 781 nt	69	++
M. tuberculosis F11	NC_009565	4 424 435 nt	65	?
M. tuberculosis H37Ra	NC_009525	4 419 977 nt	65	?
M. tuberculosis H37Rv	NC_000962	4 411 532 nt	65	?
M. tuberculosis CDC1551	NC_002755	4 403 837 nt	65	?
M. bovis BCG	NC_008769	4 374 522 nt	65	+
M. bovis AF2122/97	NC_002945	4 345 492 nt	65	++
M. leprae TN	NC_002677	3 268 203 nt	57	?

In water and in soil, the most recent common ancestor of *Mycobacteria* may have encountered free-living predators including free-living amoebae (FLA). The classification of FLA is mainly based on 18S rRNA gene sequencing (Fig. 1). These organisms are cell-wall free, unicellular eukaryotes that switch from a motile trophozoite phase towards an immobile cyst phase (Fig. 2) during starvation, drying, hypoxia and fluctuation in temperature. Trophozoites are professional phagocytes that engulf any particle with a diameter ≥0.5 μm into phagocytic vacuole with further lysosome fusion and destruction. Mycobacteria have evolved mechanisms to withdraw and resist such unicellular protist predators, including secreted toxins, and the capacity to avoid lysosomal killing and to replicate intracellularly within protozoa. Mycobacteria gained the advantage of being protected from adverse conditions by the amoebal cyst. Encystement consists in the formation of a cell wall comprising one layer as in the case of *Entamoeba*, two layers as in the case of *Acanthamoeba* and *Hartmannella vermiformis* [15], and an even more complex structure as in the case of *Giardia* [16]. In *Acanthamoeba*, the formation of the first layer (i.e. ectocyst) results in an immature cyst, and further apposition of a second, internal layer (i.e. endocyst) forms a mature cyst. The cell wall is composed of polysaccharides with β-configuration, which are cellulose in the case of *Acantamoeba* cysts [17] (Fig. 3). Cysts are resistant to alterations in osmolarity and pH, desiccation, freezing, high concentration of hydrochloric acid, moist heat, chemical antimicrobial agents and biocides [18,19]. Moreover, cysts have been shown to survive in the laboratory for at least 20 years [20]. Excystment refers to the process by which trophozoites emerge through ostioles of the amoebal cyst, after the mechanical expulsion of the opercalum [21] (Fig. 2)

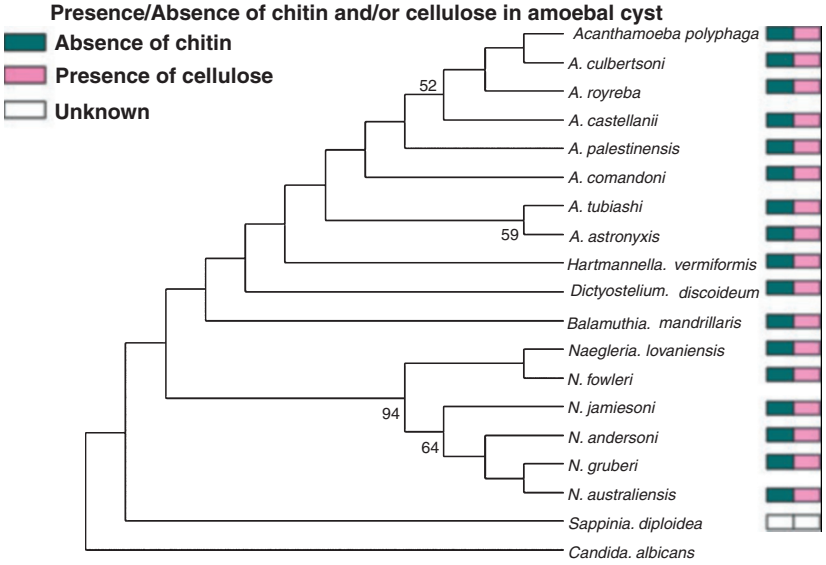

FIG. 1. Classification of free-living amoebae based on 18S rRNA gene sequencing. Only bootstrap values >50% were figured at nodes.

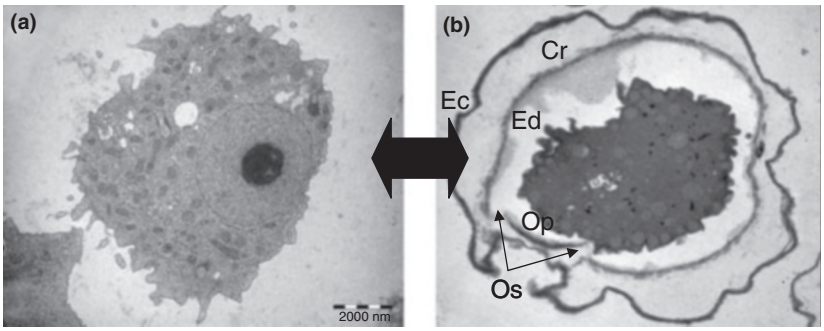

FIG. 2. Structure of *Acanthamoebae*. The trophozoites (a) measure 24–56 μm in diameter. The double walled cysts (b) measure 12–30 μm in diameter. The cellulose-containing cyst wall localized in inner endocyst. Bar, 10 μm. Os: Ostiole, Op: Opercalum, Ed: Endocyst, Ec: Ectocyst, Cr: Clear region.

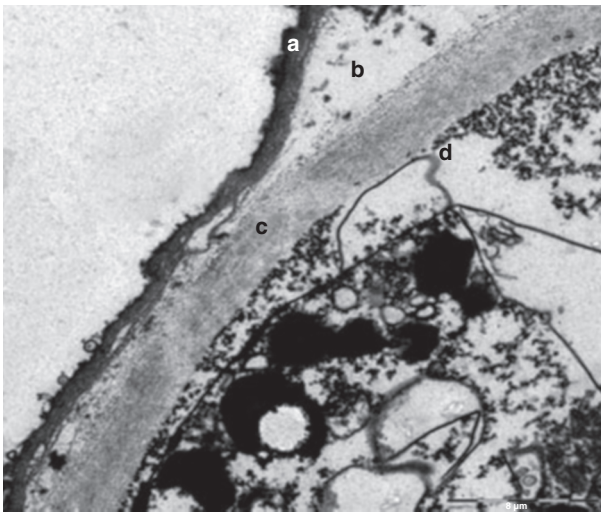

FIG. 3. Ultrastructure of *Acanthamoeba polyphaga* cyst wall. (a) Ectocyst, (b) Clear region, (c) Endocyst (cellulose), (d) Plasmic membran.

and requires the action of cellulase of the Glycosyl Hydrolase family 5 as in *Dictyostelium discodium* (Glycosyl Hydrolase family 9). Both encystement and excystement were found to be dependant on serine protease function [22].

During their evolution from the MRCA, mycobacteria have evolved strategies to avoid predation by FLA and these strategies have been further used to resist macrophage destruction, which is a key step for pathogenicity.

Mycobacteria- Free Living Amoebae Interactions (Table 2)

FLA have been isolated from habitats in common with mycobacteria [23,24] including cold drinking water systems [25,26], hot water systems in hospitals [27], cooling towers [28], and contact lens storage cases [29]. Direct observation of mycobacteria within amoebae collected in such environments has not been reported; however, mycobacteria have been isolated from FLA collected in such environments, including the isolation of 13 different *Mycobacterium* species in parallel to amoebae related to *Hartmannella*, *Echinamoeba*, *Acanthamoeba* and *Naegleria* [26]. In a hospital water network, the presence of FLA including *H. vermiformis*, *Acanthamoeba polyphaga,* and an unidentified protist was significantly correlated with the presence of mycobacteria in the same water specimen, including *Mycobacterium gordonae*, *M. xenopi* and *M. kansasii* subtype I [30]. In Benin, ten different *Mycobacterium* species, but not *Mycobacterium ulcerans* have been isolated from protected and unprotected water sources in which six different species of *Acanthamoeba*, *Naegleria* and *Tetramitus* amoeba have been detected [25] (Table 2). In most studies, waterborne mycobacteria were isolated by co-culturing a specimen with *A. polyphaga* [23,28,30] as in the case of soil mycobacteria [31]. The same approach has been used to isolate *Mycobacterium massiliense* from sputum [32] and *M. avium* subsp. *paratuberculosis* from colon biopsies collected from patients with Crohn's disease [33].

Experimental studies confirmed the reality of FLA-mycobacteria interactions. These studies used waterborne amoebae, including *Acanthamoeba polyphaga* [33–36], *Acanthamoeba castellanii* [37–41], *Hartamnella vermiformis* [42,43], and the social soil amoeba *Dictyostelium discodium* [44–48]. The ciliated protozoan *Tetrahymena pyriformis* has also been used to study the intracellular growth of the *M. avium* complex species [49]. Amoeba models allowed the study of surface receptors [50], phagocytosis [50], and bactericidal mechanisms [51], using organisms easy to cultivate with short generation times, for which specific genetic, biochemical and cell biological tools were available. *Acanthamoeba* organisms are less well characterized genetically than *D. discodium, whose* genome is available [52], but they remain viable and grow at temperature above 25°C (32–37°C), in the temperature range for natural mycobacteria-macrophage interactions [39].

TABLE 2. Environmental *Mycobacterium* species isolated by amoebal coculture

Samples	*Mycobacterium* species	Identification of endosymbionts	Amoeba line	References
Contact lens storage	*M. intracellulare*	16S rDNA	*A. lugdunensis* KA/LC6	[113]
Human gut tissue	*M. avium subsp. Paratuberculosis*	IS*900*	*A. polyphaga* CCAP 1501/18	[33]
Hospital water network	*M. xenopi, M. gordonae,M. Kansasii*	16S rDNA	*A. castellani* ATCC 30010	[30]
Sputum	*M. massiliense*	16S rDNA, *rpo*B	*A. polyphaga* Linc-AP1	[31]
Ozonated water	*M. mucogenicum*[a]	16S rDNA	*Echinamoeba exundans*	[26]
River water	*M. frederiksbergense/M. Fluoranthenivorans, M. frederiksbergense, M. gastri or M. kansasii, M. gordonae,M. insubricum, M. terrae, M. vaccae, M. vanbaalenii*	16S rDNA	*A. castellanii ATCC30010*	[26]
Sand biofilm	*M. gordonae, M. insubricum, M. poriferae*	16S rDNA	–	[26]
Sand-filtered	*M. septicum/M. peregrinum*	16S rDNA	–	[26]
Ozonated	*M. gordonae, M. fluoranthenivorans*	16S rDNA	–	[26]
Carbon biofilm	*M. fluoranthenivorans, M. gordonae, M. neglectum*	16S rDNA	–	[26]
Carbon-filtered	*M. frederiksbergense/M. fluoranthenivorans*	16S rDNA	–	[26]
Distant points	*M. anthracenicum, M. frederiksbergense/M. fluoranthenivorans, M. gadium, M. neglectum, M. vanbaalinii*	16S rDNA	–	[26]
Fountain	*M. chelonae, M. abcessus, M. monacense*	16S rDNA	*A. polyphaga* Linc-AP1	[28]
Lake	*M. neoaurum*	16S rDNA	–	[28]
Soil (Illegal dumping site, landfills)	*M. vanbaalinii, M. rhodesiae, M. frederiksbergense, M. neoaurum*	*hsp*65	*A. culbertsoni*	[31]
Swamp	*M. gordonae*[a]	16S rDNA, Culture	*Acanthamoeba* sp.	[25]
Rainwater tank	*M. malmoense*[a]	16S rDNA, Culture	–	[25]
Cooling towers	*M. chelonae, M. phocaicum, M. fortuitum, M. conceptionense, M. chiamera*	*rpo*B	*A. polyphaga* Linc-AP1	[114]

[a]Natural intra-amoebal mycobacteria.

Therefore, *Acanthamoeba* is an important member of the FLA and has served as a model organism to study the *in vitro* interactions with bacteria; for eukaryotic transcription *in vitro,* and more recently *in vivo*, this model facilitates the study of cytoskeletal and mitochondrial functions [53]. Variable multiplicity of infection (MOI) from 1:100 [34] to 1:10 [45,54] is most often used with removal of extra-amoebal mycobacteria using buffer [34], buffer in addition to antibiotic such as amikacin [37], streptomycin [44] or HCl 3% [34] (Table 3). After excystment the extraction of an intra-amoebal fraction with SDS 0.5% and culture on standard medium made it possible to obtain evidence of intracystic survival. Intra-amoebal multiplication could be measured by microscopic counting of intra-amoebal mycobacteria after Ziehl [54] (Fig. 4) or fluorescent acid-fast staining [49], electron microscopy counting [34] (Fig. 5), counting of mycobacteria colonies, or real-time PCR.

All the *Mycobacterium* species under study have been shown to be phagocytosed and to penetrate amoebal trophozoites into vacuoles, including *M. tuberculosis* [47], *M. bovis* and BCG strains [55], *M. leprae* [41,56], *M. marinum* [37,47], *M. avium* [34,37,45], *M. avium* subsp. *paratuberculosis* [33,54], *Mycobacterium kansasii* [39], *M. xenopi* [36], *Mycobacterium fortuitum* [37], *M. smegmatis* [37,38], and 26 additional non-tuberculous species [35] (Table 3). All these *Mycobacterium* species have been shown to persist in trophozoites, with the exceptions of *M. smegmatis* [37,38] and the two BCG strains (Pasteur and Japan) of *M. bovis*, which were killed within *A. castellanii* trophozoites, contrary to the *M. bovis* parent strain [55]. After phagocytosis, FLA-resisting mycobacteria grow inside trophozoites, as demonstrated for *M. avium* [34,37], *M. avium* subsp. *paratuberculosis* [33], *M. fortuitum* [37], *M. marinum* [37], *M. xenopi* [36]. Intra-amoebal growth may depend on the temperature because *M. avium* and *M. fortuitum* grew and replicated equally at 32°C and 37°C whereas *M. marinum* replicated only at 32°C and was killed at higher temperatures [37]. Only a portion of the inoculum may adapt and survive within trophozoites, as shown for *M. avium* subsp. *paratuberculosis* [33]. Likewise, the replication fate of *M. avium* within *A. castellanii* was found to vary with strains [37]. Moreover, it has been shown that inta-amoebal growth of *M. avium* increased its entry and replication within both the epithelial cell line HT-29 and human monocyte-derived macrophages, as well as its virulence in a mice model [37]. Likewise, the level of inta-amoebal replication of *M. kansasii* within *A. castellanii* correlated with the virulence of strains, regardless of the genetic subtype [39]. Measurement of intra-amoebal growth could be used as a test predictive of virulence for this opportunistic pathogen. Finally, a parachamber experiment indicated that *M. avium* may replicate saprozoically on products secreted by amoeba [34].

In further experiments, a few *Mycobacterium* species were shown to survive within the amoebal cyst, as in the case of *M. xenopi* [36], *M. avium* subsp. *paratuberculosis* [33], and 26 additional non-tuberculous species [35]. Likewise, it has been suggested that *M. bovis* was able to survive encystement [55]. Interestingly, these mycobacteria were observed in the cyst wall and, more precisely, the exocyst layer of the cyst. An

TABLE 3. Experimental model of interaction between mycobacteria and protists

Mycobacterium sp.	Protozoan line	Evidences of Intra-amoebal		Extra-cellular mycobacteria treatment	Cystic location	Reference
		Survive	Multiplication			
M. abscessus	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	Am 100 μg/2 h	NR	[35]
M. aurum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. bohemicum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. bovis	*A. castellanii* CCAP 1501/1A	Trophozoïte/Cyst	–	–	NR	[55]
M. bovis BCG	*A. castellanii* CCAP 1501/1A	–	–	–	NR	[55]
M. chelonae	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. fortuitum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. fortuitum	*A. castellanii* ATCC 30234	Trophozoïte	CFU counts	–	NR	[37]
M. fortuitum	*A. castellanii*	Trophozoïte	NR	NR	NR	[40]
M. gastri	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	Am (100 μg/2 h)	NR	[35]
M. goodii	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. gordonae	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. immunogenum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. kansasii	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. kansasii	*A. castellanii* ATCC 30010	Trophozoïte	LM (Z.N)	NR	NR	[39]
M. lentiflavum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	Am (100 μg/2 h)	NR	[35]
M. leprae	*A. culbertsoni* ATCC 30171	Trophozoïte	NR	NR	NR	[41]
M. mageritense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	Am (100 μg/2 h)	NR	[35]
M. malmoense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. marinum	*A. castellanii* ATCC30234	Trophozoïte	CFU counts	NR	NR	[37]
M. marinum	*A. castellanii*	Trophozoïte	NR	NR	NR	[40]
M. marinum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	Am (100 μg/2 h)	NR	[35]
M. massiliense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	NR	NR	[32]
M. mucogenicum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. peregrinum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. phlei	*A. castellanii*	Trophozoïte	NR	–	NR	[40]
M. porcinum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. septicum	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. simiae	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. simiae	*A. castellanii*	Trophozoïte	NR	–	NR	[40]
M. smegmatis	*A. castellanii*	Trophozoïte	NR	NR	Ectocyst	[38]
M. smegmatis	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	Am (100 μg/2 h)	NR	[35]
M. smegmatis	*A. castellanii* ATCC30234	Trophozoïte/Cyst	CFU counts	NR	NR	[37]
M. szulgai	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. terrae	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. tusciae	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. ulcerans	*A. castellanii*	Trophozoïte	NR	NR	NR	[25,40]
M. xenopi	*A. polyphaga* Linc-API	Trophozoïte/Cyst	LM, EM, qPCR	NR	NR	[36]
M. marinum	*D. discodium* AX2	Trophozoïte	CFU counts	Streptomycin	NR	[44]
M. scrofulaceum	*T. pyriformis* ATCC30202	Trophozoïte/Cyst	CFU counts/FAM	PBS watching	NR	[49]
M. a. a	*D. discodium* AX2	Trophozoïte	CFU counts	Am (200 μg/2 h)	NR	[45]
M. intracellulare	*T. pyriformis* ATCC30202	Trophozoïte/Cyst	CFU counts/FAM	PBS watching	NR	[49]
M. avium	*T. pyriformis* ATCC30202	Trophozoïte/Cyst	CFU counts/FAM	PBS watching	NR	[49]
M. avium	*A. polyphaga* ATCC 30872	Trophozoïte/Cyst	CFU counts	HCl (3%)	Ectocyst	[34]
M. avium	*A. castellanii* ATCC 30234	Trophozoïte/Cyst	CFU counts	Am (100 μg/2 h)	NR	[37]
M. a. a	*A. polyphaga* Linc-API	Trophozoïte/Cyst	NR	–	NR	[35]
M. a. p	*A. polyphaga* CCAP 1501/18	Trophozoïte/Cyst	qPCR	PBS watching	NR	[33]
M. a. p	*A. castellanii* CCAP 1501/1B	Trophozoïte	CFU counts	NR	NR	[54]
M. a. p	*A. castellanii* CCAP 1501/3B	Trophozoïte	CFU counts	NR	NR	[54]
M. intracellulare	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)		Present study [35]
M. chimaera	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)		–
M. colombiense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)		–
M. marseillense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)		–
M. timonense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)		–
M. bouchedurhonense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)		–
M. arosiense	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)		–
M. avium	*A. polyphaga* Linc-API	Trophozoïte/Cyst	qPCR/EM	HCl (3%)	Ectocyst	–

Am, Amikacine (100 μg/2 h); NR, Not Reported; CFU, Colony Forming Unity; *M. a.a*, *Mycobacterium avium* subsp. *avium*; *M. a .p*, *Mycobacterium avium* subsp. *paratuberculosis*; EM, Electronic Microscopy; LM, Light Microscopy; ZN, Zhiel Nelseen staining; FAM, Fluorescent acid-fast microscopy.

increased oxygen uptake during the first 10 h of *Hartamnella* encystment has been shown; subsequently, oxygen consumption gradually decreased to an immeasurable value when the cyst formation was complete [57]. In fact, a strict hypoxic condition can be tolerated by some free-living protists but many of them tolerate a very low level of oxygen. For example, it has been shown that the strict, non-sporulated bacterium *Mobiluncus curtisii* was able to grow within *Acanthamoeba culbertsoni* and other *Acanthamoeba* strains which were maintained under an aerobic atmosphere [58]. This anaerobic condition during a long period of time (more than 20 years) may be favourable to intracellular mycobacteria, which could survive in dormancy, and the amoebal cyst could be used as a model to study this particular state of mycobacteria. Indeed, despite the fact that *M. tuberculosis* is an aerobe bacterium, it can survive extended periods of anaerobiosis by induction of over 60 genes known as the dormancy regulon, also activated during human latent infection [59].

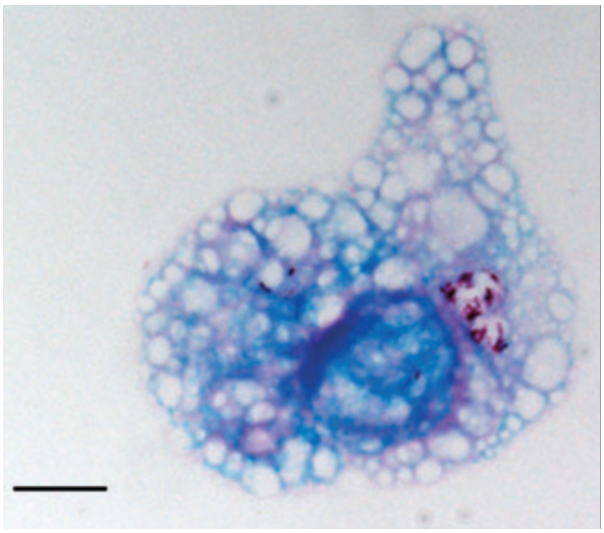

FIG. 4. Ziehl staining of infected *Acanthamoeba polyphaga* by *M. colombiense* after 24 h of incubation at 32°C (Gr × 1000; bar = 10 μm).

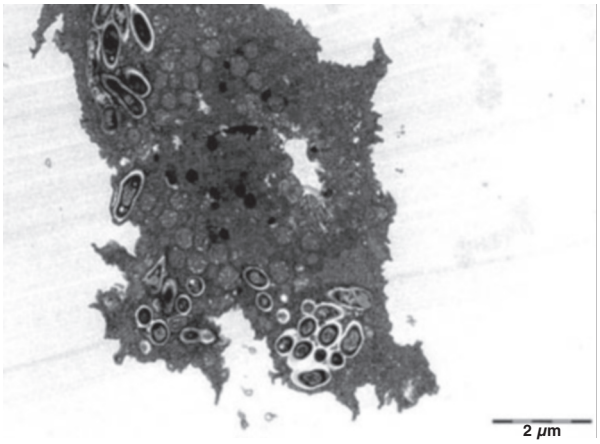

FIG. 5. Transmission electon microscopy of infected *A. polyphaga* trophozoites 24 h post infection. Cluster of *M. colombiense* bacilli were located within several vacuoles per cell. EM micrograph showing a social phagosome (containing more than one bacterium) containing a dividing bacterium. B. EM micrograph of mature of *A. polyphaga* cyst containing *M. colombiense* (arrow) within cell wall (note that the outer cell wall is divided and surrounds the bacterial cells). Bar = 2 μm.

Free-living Amoebae: Models for Macrophage-Mycobacterium Interactions

The observation of FLA-mycobacteria interactions contributed to the understanding of the mechanisms that sustain the resistance of mycobacteria to the human macrophage used to study host-mycobacteria interactions [60,61]. Regarding the entry of mycobacteria using the complement receptors CR1, CR3 and CR4, and the manose receptor, it has been observed that *M. avium* mutants lacking a pathogenicity island exhibited highly similar defects in invading *A. castellanii* and human macrophages [62]. In this model, 20 genes initially found to be up-regulated in trophozoites, were further found to be also up-regulated in human macrophages; these genes encoded metabolic pathways, protein transcription and translation and macromolecule degradation [63]. Also, *M. xenopi* was found to survive and to multiply within *A. polyphaga* trophozoites [36] and was found to survive and to replicate, ten-fold in 48 h, within human peripheral macrophages [64]. Likewise, it was observed that *A. castellanii* phagosomes containing *M. smegmatis* fused with lysosomes as soon as 5 min post-inoculation, whereas the majority of mycobacteria are partially degraded 30 min post-inoculation in the absence of persistence [37]. This has been confirmed in a further experiment indicating that a double mutant lacking the *mspA* and *mspC* porin genes exhibited enhanced persistence, demonstrating the role of mycobacteria outer membrane permeability in intracellular persistence [38].

Survival within amoebal trophozoites may train environmental mycobacterial species to adapt and survive within the hostile human macrophages by avoiding degradation by the host cell lysosome. After pathogen uptake by phagocytosis, the host cell enters into lag phase that reflects the early and active transformation of phagosomes containing pathogens into real replication niches, implicating the protein MAG24 [65] (Fig. 6) (Table 4). *M. tuberculosis, M. bovis*, *M. marinum* and *M. avium* have been shown to prevent the maturation of the phagosome into a phagolysosome, thus residing in a replicative early phagosome [66] .

Fine electron microscopy observations indicated that mycobacteria prevented phagosome maturation only when they established and maintained a close apposition with the phagosome membrane, which is probably required for the inhibition of the phagosome conversion [67], a situation seen only when mycobacteria enter as isolated, non-clumped organisms [68]. In *D. discoideum*, phagosome maturation takes place 90 min after uptake of mycobacteria, and includes a sequence of events beginning with the accumulation of vacuolar H+ATPase (VatA), the endosomal protein p80, and the vacuolin (VacB) [65] (Table 4). Further gradual accumulation of VacB surrounding cathepsin D is followed by morphological changes in the replication niches and enhanced proliferation of the pathogen [65]. In these experiments, *M. marinum* has been used as a model organism for *M. tuberculosis* [44], being closely related [3]. In the course of *M. marinum* infection, granulomas are indistinguishable from those caused by *M. tuberculosis* [69] and the intracellular proliferation niches

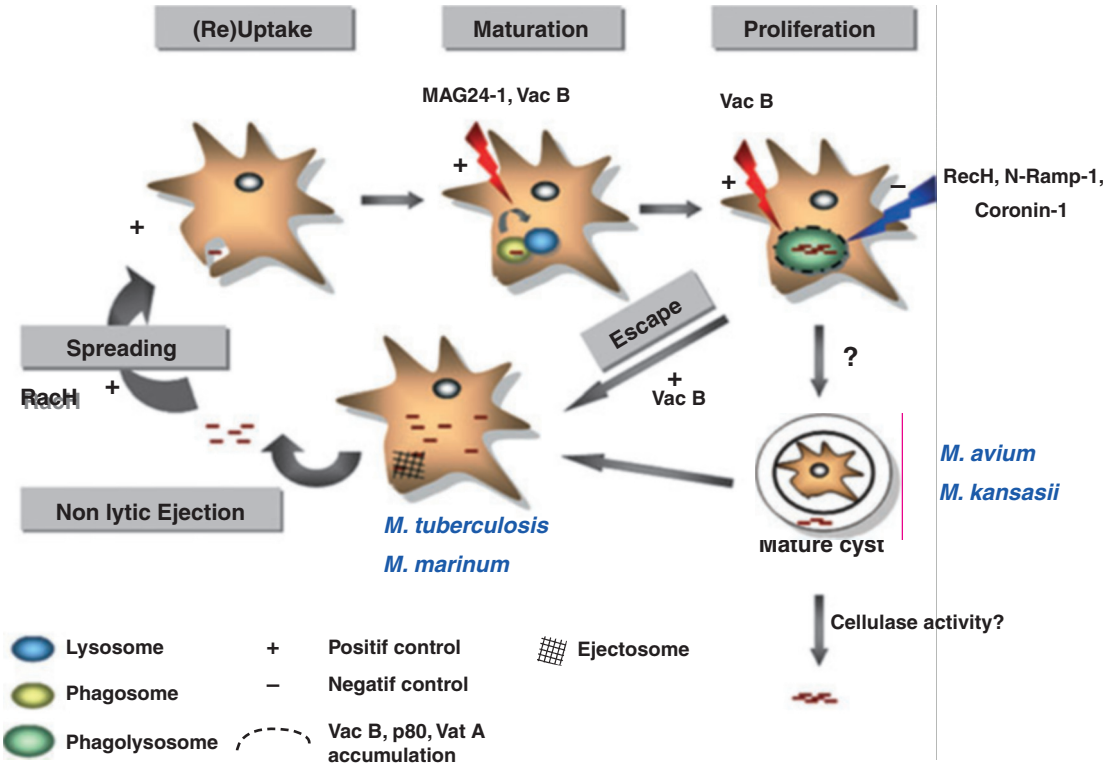

FIG. 6. Mechanism of interaction between free-living amoeba and *Mycobacterium* sp.

TABLE 4. Molecular determinants implicated in *Mycobacterium*-amoeba interactions. *D. discodium*: *Dictyostelium discodium*

Molecular determinants	Microorganism	Mechanisms	References
II. Mycobacterial determinants			
Porins (*MspA*)	*M. smegmatis*	Rise of cell wall Permeability → Reduce intracellular persistence Role in uptake by environmental amoeba	[38]
PI (Pathogenicity Island)	*M. avium*	Mutation → Reduce growth in *D. discodium* cells	[62]
mag-24-1 (PE-PGRS) family	*M. marinum*	Control the multiplication in host cells	[44]
II. Amoebal determinants			
N-Ramp1: metal transporter in phagosomal membrane	*D. discodium*	Loss → Enhance replication of ingested bacteria	[115]
RacH, RacH GTPase	*D. discodium*	Control endosomal membran trafficking Phagosomal acidification Regulate the actin cytoskeleton	[116]
Vacuolin B (Flotillin-like protein)	*D. discodium*	Cell-to-cell spreading of mycobacteria infection Role in growth in vacuole and release of mycobacteria into cytosol	[65]
Vacuolar H^+-ATPase (Vat A)	*D. discodium*	Control the endocytic pathway	[65]
P80	*D. discodium*	–	[65]
Coronin (Actin binding protein)	*D. discodium* mutant	Absence → Enhanced survival of *M. marinum*	[44]
Diffusible factor	*Acanthamoeba*	Promote growth of *Mycobacterium*	[34]

of both organisms are very similar [70]. Several *M. marinum* virulence mutants have phenotypes similar to *M. tuberculosis* mutants and can be completed by *M. tuberculosis* genes [71–73]. The most prominent characteristics of the *M. tuberculosis* phagosome are its incomplete luminal acidification [74] by exclusion of the vacuolar proton H+-ATPase [74] and the absence of mature lysosomal hydrolases. The accessibility to transferrin-bound iron [74] and trafficking of transferrin receptors through mycobacterial phagosome [75] indicate that the mycobacterial phagosome is not a static organelle, despite the blockade in the acquisition of lysosomal compartments. Iron supply by the transferrin is required for *Mycobacteria* replication [76]. *M. tuberculosis* manipulates the small rab GTPase function to colonize the host [77]. Maturation of mycobacterium-containing phagosomes is blocked at the Rab5-positive stage (early phagosome). Contrary to the retention of the small GTPase Rab5, mycobacterial phagosomes do not recruit Rab7, a molecule required for phagosome conversion. Rab5 and Rab7 are central players in the biogenesis of the endosomal pathway [78]. Because phagosome maturation is blocked at the Rab5-stage, Rab5 effectors, which are involved in the phagosome maturation

process, are recruited into mycobacterial phagosomes, with the notable exception of a Rab5 effector early endosomal autoantigen 1 (EEA1) [79,80].

Moreover, it has been shown that phosphatidylinositol 3-phosphate (PI3P), a critical regulator of Rab5 effector recruitment, such as EEA1 [81], is reduced or absent in phagosomes containing *M. tuberculosis* [82]. PI3P is generated by the cellular enzyme VPS34, a type III phosphatidylinositol 3-kinase (PI3K) [83]. PI3P generation is inhibited by two *M. tuberculosis* products, including the lipid liparabinomannan (LAM) [77] and a PI3P phosphatase SapM [84]. The inhibition of PI3P generation by LAM and SapM represents a double mechanism required to 'hijack' the maturation of the *M. tuberculosis*-containing phagosomes, thus minimizing the possibility of PI3P-dependent maturation into phagolysosome.

It has also been reported that *M. tuberculosis,* as well as *M. marinum* and *M. leprae,* could escape from the phagosomes and grow within the cytosol of host cells; however, it is not required for replication [85,86]. The efficient translocation from vacuole to cytosol depends on an intact region of difference (RD), one locus [86,87] which encodes a type 7 secretion system and essential secreted effectors [88]. The effector ESAT-6 was demonstrated to be secreted by the ESX-1 secretion system and to be responsible for breakage of the replication niche and pore forming which facilitate the exit of *M. marinum* and *M. tuberculosis* into the cytosol [87]. Intracellular growth of mycobacterial species involves a unique trafficking pathway, which blocks maturation of the phagolysosome and establishes a replicative compartment that maintains vesicular contact with the plasma membrane surface [89]. This mechanism of persistence to phagocytosis of some *Mycobacterium* species, such as *M. avium* or *M. marinum,* in both amoebal and mammalian cells, has put into question the role of some host cell proteins. One crucial protein was coronin/TACO (Tryptophan aspartate-containing coat protein), a phagosomal protein implicated in intracellular mycobacterial trafficking through the inhibition of phagolysosme fusion and the activation of mycobacterial growth [90]. Mutation in the coronin gene permitted the isolation of strains defective in particle phagocytosis, fluid phase uptake, migration, and cytokinesis [91]. However, the *M. marinum*/*D. discoideum* model showed that the absence of coronin did not enhance intra-amoebal replication, suggesting that other amoebal factors were required for intra-amoebal replication of *M. marinum* [44]. Further host factors important in restricting mycobacterial growth include the NRAMP-1 transporter on the phagosome, presumably due to limitation of divalent cations within this compartment [92]. The flotillin homologue vacuolin B and p80, a predicted copper transporter, accumulates at the vacuole during pathogen replication until it finally ruptures and the bacteria are released into the host cytosol [65]. Flotilin-1 accumulation at the replication niche and its rupture were also observed in human blood monocytes. Infection of various *Dictyostelium* mutants demonstrates that the absence of one of the two *Dictyostelium* vacuolin isoforms renders the host more immune to *M. marinum*. Conversely, the absence of the small GTPase RacH renders the host more susceptible to *M. marinum* proliferation but inhibits cell-to-cell spreading [65] (Table 4).This protein factor was implicated also in regulation of the actin cytoskeleton and in endosomal membrane trafficking and acidification. Like *M. tuberculosis*, *M. marinum* evades the endocytic pathway after uptake by host cells [70], and factors associated with the intracellular growth of this organism also appear to play a role in *M. tuberculosis* pathogenesis [93]. Recently, it has been discovered that *M. tuberculosis* and *M. Marinum,* but not *M. avium*, were ejected from the *Dictyostelium* trophozoite, owing to an actin-based structure, the ejectosome. This conserved nonlytic spreading mechanism requires a host cytoskeleton regulator and an intact mycobacterial ESX-1 secretion system [47]. The mycobacterium-induced blockage of phagosome-lysosome fusion is an essential prerequisite for such escape into the cytosol. Escape of mycobacteria outside the phagosome occurs after a significant delay [86] and requires the secreted effector ESAT-6, recently linked to niche breackage and pore forming activity [87]. This phenomenon has been well described for *M. marinum*, which escapes outside the phagosome and is propelled by actin-based motility into the cytoplasm [85]. The coronin mutants of *D. discodium* were used to determine the genetic basis of mycobacterial trafficking in mammalian macrophages [44]. Vacuolar escape has been linked to the ESX-1 secretion system locus. Moreover, *M. marinum* undergoes actin-based motility in the cytosol, allowing the spread of the organizm to neighbour cells [85]. Wiskott-Aldrich syndrome family proteins are necessary for this actin-based motility and intercellular spreading [85,94]. However, escape into the cytosol does not seem to be required for mycobacterium replication, contrary to what has been observed for *Listeria monocytogenes* [95].

Conclusions and Perspectives

Among several species of free-living amoebae, only members of the genus *Acanthamoeba*, *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia diploidea* are the opportunistic pathogens [96] mainly responsible for keratitis [97] and encephalitis [98]. Moreover, FLA, including 'Limax amoeba', *A. polyphaga*, *A. castellanii*, *Hartmanella vermiformis* and *Naegleria gruberi,*

with a composition comparable to that of inanimate environment of the individuals have been isolated from the human nasal mucosa [99]. *Hartmanella* species have been also isolated from human throats [100]. Among these FLA demonstrated to infect humans, *Acanthamoeba* spp. are known to host the facultative and obligate bacterial pathogens [26] *Parachlamydiae* [101], *Salmonella typhimurium* [102], *Escherichia coli* O157 [103], *Francisella tularensis* [104], *Burkholderia pseudomallae* [105], *Coxiella burnetii* [106], *Helicobacter pylori* [107], *Legionella pneumophila* [108], *Chlamydia* [109], *Vibrio parahaemolyticus* [110] as well as the fungus *Cryptococcus neoformans* [111]. This review indicates that all mycobacteria known to be obligate or facultative human pathogens should be added to the growing list of amoeba-resistant bacteria [112]. Such amoeba-resistant bacteria have been recovered from human nasal swabs using amoebal co-culture [112]. One could therefore hypothesize that FLA may be vectors for mycobacteria and may facilitate their penetration into the host and facilitate their pathogenic role, as was suggested more the 30 years ago for *M. leprae* [41], with recent experimental confirmation [56]. Indeed, intra-amoebal location has been demonstrated to exacerbate the virulence of *M. avium* [37] and to protect *M. avium* and *M. xenopi* against antibiotics and biocides [35,54]. Environmental and clinical studies are warranted to further test this hypothesis.

Transparency Declaration

All co-authors report that they have no conflicts of interest relevant to this article.

Methods for Literature Review and Selection

We performed the bibliographic research in the PubMed database by using the following keywords: interaction between amoeba and *Mycobacterium*, amoeba and *Mycobacterium* coculture, association of *Mycobacterium* and amoeba, survival of mycobacteria in amoeba. We then selected English publications published in the period 1967–2009.

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