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MEETING REVIEW

The Many Lives of Nontuberculous Mycobacteria

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ABSTRACT Nontuberculous mycobacteria (NTM) include species that colonize human epithelia, as well as species that are ubiquitous in soil and aquatic environments. NTM that primarily inhabit soil and aquatic environments include the *Mycobacterium avium* complex (MAC) (*M. avium* and *Mycobacterium intracellulare*) and the *Mycobacterium abscessus* complex (MABSC) (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*) and can be free living, biofilm associated, or amoeba associated. Although NTM are rarely pathogenic in immunocompetent individuals, individuals who are immunocompromised, due to either an inherited or acquired immunodeficiency, are highly susceptible to NTM infection (NTMI). Several characteristics, such as biofilm formation and the ability of select NTM species to form distinct colony morphotypes, all may play a role in pathogenesis that is not observed in the related, well-characterized pathogen *Mycobacterium tuberculosis*. Different morphotypes of NTM have been recognized and characterized since the 1950s, but the mechanisms that underlie colony phenotype change and subsequent differences in pathogenicity are just beginning to be explored. Advances in genomic analysis have led to progress in identifying genes important to the pathogenesis and persistence of MAC disease as well as in illuminating genetic aspects of different colony morphotypes. Here we review recent literature regarding NTM ecology and transmission, as well as the factors which regulate colony morphotype and pathogenicity.

KEYWORDS amoeba, *M. abscessus*, *M. avium*, *M. intracellulare*, morphotype, mycobacteria, nontuberculous mycobacteria, tuberculosis

Nontuberculous mycobacteria (NTM) are a subset of *Mycobacterium* species that are found in many environmental niches in nature. Although NTM are harmless to most individuals, each year (at both the national and international levels) there are increasingly more individuals diagnosed with NTM infection (NTMI). Clinical presentations of NTMI include pulmonary infection, disseminated infection, skin disease, and lymphadenitis. Although risk factors for NTMI include immunodeficiency or underlying barrier dysfunction, most NTMI patients do not have any known risk factors. This is especially true of children with NTMI, in whom disease manifests primarily as a distressful and disfiguring cervical lymphadenitis. It is important to have a basic understanding of the host and bacterial factors that maintain human-NTM commensalism, as their perturbation may cause an infection to progress at the expense of the human host (1).

NTM include species that colonize human epithelia, as well as species that are found in soil and aquatic environments. The NTM species that colonize human epithelia are largely nonpathogenic and can be found on skin (2–4) and along the genitourinary (5–7), gastrointestinal (7), and respiratory (8–14) tracts. The NTM species that are found in soil and aquatic environments include *Mycobacterium vaccae*, the *Mycobacterium avium* complex (MAC), and the *Mycobacterium abscessus* complex (MABSC) (15, 16). Although most NTM are traditionally considered to be opportunistic pathogens, *M. vaccae* is unique in that it is also a transient human colonizer (17) and benefits the host

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in a manner that resembles ecological mutualism: *M. vaccae* inhibits pulmonary allergic inflammation in mice (18), as well as decreases anxiety in both mice (19) and humans (20), via an as-yet-undefined gut-brain-microbiota axis (21). MAC was first isolated from wood pigeons (22) but is now known to be ubiquitous in the environment and found in freshwater, salt water, soil, food, dust, and domestic and wild animals (23–26). In both water and soil, MAC and MABSC species (MAC/MAB) can be free living, biofilm associated, or amoeba associated (27, 28). Infection with MAC/MAB can follow exposure to aerosols of MAC/MAB-containing water while bathing (29, 30) or to aerosols of MAC/MAB-containing soil while gardening (31) or during a natural disaster (32). NTM therefore occupy a unique and broad ecological niche and necessarily exhibit a remarkable range of genetic adaptations to their varied environments.

With an ever-growing number of mycobacterial species being identified, the importance of understanding the interspecies relationships as well as the roles of individual species in human colonization and pathogenesis cannot be overlooked. Here we review the diversity, evolution, and genetic relationships between existing mycobacterial species, the environments in which they are found (specifically, their niche as aquatic organisms as well as their interactions with environmental amoebas), and the current research on NTM colony morphotypes.

THE DIVERSE LIVES OF NONTUBERCULOUS MYCOBACTERIA

The nomenclature and classification of mycobacteria has remained unchanged for most of its known history (33). Species are generally grouped into three major categories based on propensity for human infection: obligate pathogens (e.g., *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium marinum*, and *Mycobacterium ulcerans*), facultative or opportunistic pathogens (e.g., *M. avium*, *Mycobacterium intracellulare*, *M. abscessus*, and *Mycobacterium kansasii*), and strictly commensal or saprophytic bacilli (e.g., *Mycobacterium smegmatis*, *Mycobacterium vanbaalenii*, and *Mycobacterium thermoresistibile*) (34). There are more than 170 recognized species of mycobacteria, with more being added on a regular basis (35). Until recently, the phylogenetic relationships of mycobacterial species were based largely on 16S rRNA sequencing; however, the increased availability and cost-effectiveness of whole-genome sequencing (WGS) has led to more mycobacterial species being sequenced, as well as more robust comparative genomics on which to base phylogenetic relationships. Phylogenies based on WGS data are generally in concordance with those based on 16S rRNA data. Namely, rapid-growing mycobacteria ("rapid growers") and slow-growing mycobacteria ("slow growers") are clearly separated, with the rapid growers being more ancestral relative to the slow growers and the slow growers (i.e., more commonly pathogenic mycobacteria) being a distinct evolutionary branch. Where differences do exist between WGS- and 16S rRNA-based phylogenies is in regard to *M. leprae* (36). Some WGS-based phylogenetic trees resemble those of 16S rRNA-based trees, placing *M. leprae* more closely related to the NTM species *M. avium* (37), whereas others have placed it as a close sister clade to *M. tuberculosis* (38, 39). Many of these differences arise depending on the type of analysis used and the stringency of the thresholds used for software packages. Although these differences will likely continue to be a topic of debate, the overall structure of the mycobacterial phylogenetic tree is relatively accepted and well characterized (38).

Whole-genome sequencing of NTM has increased our understanding of the genetic evolution of the mycobacterial family. The recently sequenced *Mycobacterium terrae* complex has been placed as an intermediate group between rapid- and slow-growing mycobacteria, representing an evolutionary link for the growth rate shift (38). High gene turnover rates have been observed in the evolutionary timeline of NTM (39), and the number of 1:1 orthologs between sequenced NTM genomes appears to be very low (38). Even though NTM species may share a similar number of genes, the presence of species-specific genes appears to be very high and diverse (40). The role of horizontal gene transfer in mycobacteria is debated, with some studies reporting a very low impact on mycobacterial evolution, evidenced by the low number of transposable

elements present in mycobacterial genomes (38). Other studies, however, report a larger role for horizontal gene transfer based on sequence similarities and genomic islands between species (41). A recent annotation of multiple NTM genomes demonstrated that a majority of predicted genes could not be assigned a specific function (38).

Importantly, mycobacterial genome comparisons have led to a novel model regarding the evolutionary divergence of NTM and obligate pathogenic mycobacteria: due to the relatively small genome sizes of the obligate pathogens *M. leprae* and *M. tuberculosis*, the evolution of human pathogenicity corresponded to a large loss of ancestral genes with a gain of several new genes more adapted to an obligate intracellular lifestyle (40, 42). *M. leprae* and *M. tuberculosis* are remarkably distinct from one another in terms of evolution: whereas *M. leprae* evolved ~14 million years ago (43), the evolution of human-adapted *M. tuberculosis* was more recent, ~10 thousand to 70 thousand years ago (44), and it was possibly dispersed to New World populations via migratory seals and sea lions (45). What *M. leprae* and *M. tuberculosis* have in common, however, is that in contrast to nearly all NTM, which must survive in soil and aquatic environments outside a living host, they do not exist as free-living organisms in nature. Based on their biologies, *M. leprae* and *M. tuberculosis* would therefore have less need for gene regulation and adaptive responses to the environment than NTM that are found in the environment. In addition to having smaller genome sizes, pathogenic mycobacteria also have genes enriched in DNA repair and recombination mechanisms, while opportunistic pathogens have an enrichment in membrane transport genes which aid in nutrient uptake and drug efflux (40). The genes responsible for energy metabolism appear to be more NTM species specific, owing to the fact that each NTM species must adapt to its own soil or aquatic environment (40).

THE AQUATIC LIVES OF NONTUBERCULOUS MYCOBACTERIA

Aquatic environments can be significant NTM reservoirs, and water is increasingly recognized as an important NTM transmission medium. The aquatic environments in which NTM reside can be as small as a showerhead or as large as a watershed (46). The aquatic microenvironments in which NTM have been found are largely of human origin: NTM-containing water supplies have been responsible for outbreaks of NTM disease in hospitals (47, 48), as well as for outbreaks among footbath customers (49, 50), metal workers (51, 52), and alternative-medicine users (53). Heating water can be insufficient to clear NTM from water supplies, as several NTM species resist water temperatures up to 55°C (a temperature at which *Legionella pneumophila* is heat susceptible), whereas other NTM species can resist water temperatures up to 70°C, with *Mycobacterium xenopi* being the most thermoresistant (54). Environmental sampling revealed that mycobacteria comprised 1/3 of all microbes in the water of an indoor pool and 8/10 of all microbes in the surrounding bioaerosol (55). The aquatic macroenvironments in which NTM have been found include those of human origin (e.g., municipal water supplies) (56–59) but also natural watersheds (46) and lakes (60, 61). Data from experimental models suggest that the water flow in aquatic micro- and macroenvironments is insufficient to prevent the formation of NTM biofilms (62), which facilitate NTM survival in numerous hostile environments (63).

Not surprisingly, NTM are well adapted to their aquatic niche and appear to have minimal requirements to survive as free-living organisms in water, as evidenced by the ability of NTM to survive in sterile deionized water for over 1 year (64), as well as in lake water under hypoxic conditions (60, 61). In their natural environment, NTM are often presumed to exist as free-living or biofilm-associated organisms; however, this seems at odds with the concept of mycobacteria being intramacrophagic pathogens. For this reason, increasing attention is being paid to the roles of environmental amoebas in sustaining NTM in the environment (28). The concept of intra-amoebae bacteria being a source of disease is accepted in the field of Legionnaires' disease and is increasingly recognized in the field of mycobacteriology since the first demonstrations that NTM-amoeba interactions promote NTM virulence (65, 66).

Acanthamoeba is a genus of free-living amoebas that have been found in a wide

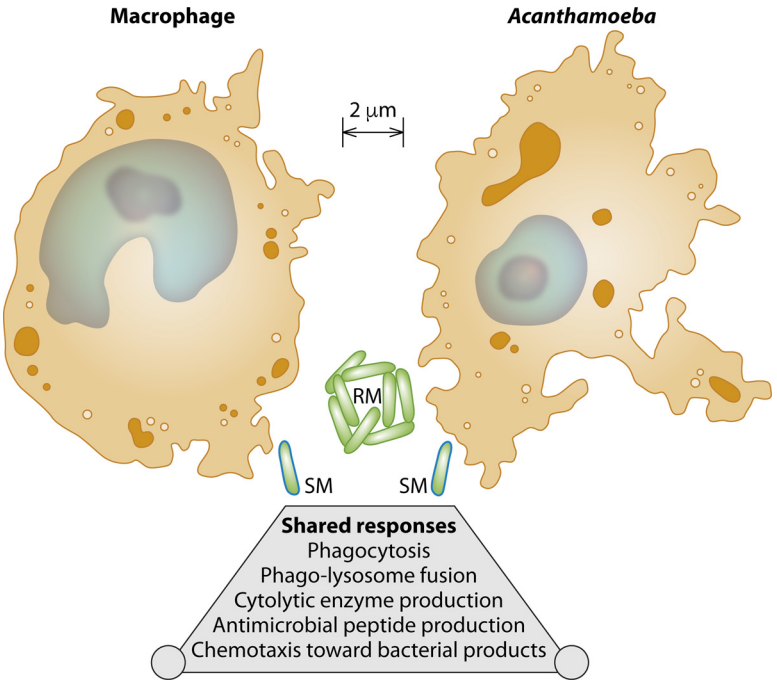

FIG 1 Macrophages and *Acanthamoeba* species are similar in their morphology and responses to bacteria. Depicted are the cell morphologies and relative sizes of three cell types (macrophages, *Acanthamoeba*, and *Mycobacterium*), based on the electron microscopy images and measurements of Lei et al. (macrophages) (129), Gonzalez-Robles et al. (*Acanthamoeba*) (72), and Schoonmaker et al. (*Mycobacterium*) (130). Macrophages and *Acanthamoeba* spp. resemble one another in size and cell morphology: both are eukaryotes with cytoplasm (orange), intracellular vesicles (brown), a nucleus (light blue), and a nucleolus (dark blue). Smooth-morphotype (SM) mycobacteria (light green) can exist as singular bacilli due to the presence of surface glycopeptidolipids (GPLs) (dark blue outline); rough-morphotype (RM) mycobacteria (light green) can exist as multicellular aggregates due to loss of GPL transport and synthesis (represented by the absence of a dark blue outline). In *M. abscessus*, these GPL transport and synthesis genes include *mps1*, *mbtH*, and *mmpL4b* (120); however, there are also GPL-independent mechanisms underlying the smooth morphotype, as this phenotype and SM→RM transitions occur in NTM species that do not produce GPL (114). Upon encountering bacteria in their respective environments, macrophages and *Acanthamoeba* have shared responses: phagocytosis, phagolysosome fusion, production of cytolytic enzymes and antimicrobial peptides, and chemotaxis toward bacterial products.

range of soil and aquatic environments, including city dust (67), bottled mineral water (68), eyewash stations (69), the waterlines of dental water flossers (70), and Antarctic soil and water (71). *Acanthamoeba* spp. are bacterivores, and their life cycle involves transitioning between encysted and trophozoite forms (72). Whereas the encysted form of *Acanthamoeba* resembles a capsule with a coarse and furrowed surface, the trophozoite form is oddly reminiscent of vertebrate macrophages, in terms of both morphology and cell biology (Fig. 1). Specifically, *Acanthamoeba* trophozoites chemotax toward bacterial products (73), release antimicrobial peptides (74), and engulf bacteria in phagosomes that subsequently undergo phagolysosomal fusion (75–77). *Acanthamoeba* trophozoites also affect bacterial lysis by expressing a broad range of cytolytic enzymes: lysozyme (78), serine and cysteine proteases that are active over a wide pH range (79), α- and β-glucosidases, β-galactosidase, β-*N*-acetylglucosaminidase, amylase, and peptidase (80). Also, similar to the case for vertebrate macrophages, the extent to which *Acanthamoeba* phagolysosomal fusion kills mycobacteria is mycobacterial species dependent (72, 81).

Numerous NTM species can enter *Acanthamoeba* during the trophozoite phase and survive within cysts for prolonged periods (82). Specific *Acanthamoeba* species that are known to be parasitized by NTM are *Acanthamoeba griffini*, *Acanthamoeba polyphaga*, and *Acanthamoeba castellanii*. *Acanthamoeba griffini* is a halophilic species that was first isolated from seawater (83) and is now known to inhabit marine environments (84), hot springs (85), air conditioners (86), and contact lenses (79). In contrast, *A. polyphaga* is

freshwater associated and was first isolated from a pond (83). NTM residing within *A. polyphaga* cysts can survive for over 2 weeks and are more resistant to the germicidal effects of chlorine (82). *Acanthamoeba castellanii* resides in soil, marine, and freshwater environments (83, 87). For any aquatic environment, the relative proportion of free versus amoeba-associated NTM is unknown; however, there are numerous reports demonstrating the ability of NTM to persist within *Acanthamoeba* in experimental systems (65, 81, 82, 88–91). These experimental data support the possibility that amoebal encystation allows NTM to persist in aquatic environments for extended periods. This is important, because growing *M. avium* in amoebas enhances their infectivity and virulence in a mouse infection model (compared to *M. avium* grown in the absence of amoebas) (65, 91).

Researchers are leveraging the possibility of an *Acanthamoeba* host to better understand how differences in bacterial genomes influence NTM survival. *Acanthamoeba* coculture systems have been used to collect virulent NTM isolates, identify conserved pathogenesis mechanisms, and make predictions regarding NTM transmission (81, 92–94). Just as the genetic tractability of another amoeba (*Dictyostelium discoideum*) has been used to discover how tubercular mycobacteria exit a cell (95), so too can novel methods of NTM transposon mutagenesis (96–99) be used to identify genes that augment or inhibit intra-*Acanthamoeba* survival. Once such genes have been identified, their presence in the genomes of virulent NTM isolates can be assayed and used to support a model wherein intra-amoeba survival is a prerequisite for intramacrophage survival. Collectively, existing data from *Acanthamoeba* coculture models support an intriguing concept that has already been applied to *Legionella* (100): that the resemblance of *Acanthamoeba* trophozoites to macrophages may naturally select for NTM bacilli that are adapted to survive within macrophages, thus increasing their fitness for intramacrophage survival in human hosts.

THE SMOOTH AND ROUGH LIVES OF NONTUBERCULOUS MYCOBACTERIA

Research on mycobacterial virulence factors has understandably focused on *M. tuberculosis* for many years; however, the emergence of NTM as globally significant pathogens (101–106) and their increasing prevalence in immunocompetent hosts (107, 108) have given rise to the need to further understand pathogenesis in a broader range of mycobacterial species. An excellent review of *M. tuberculosis* virulence factors and their corresponding roles in NTM was recently published (109). While the small genome size, large number of pseudogenes, and unculturable nature of *M. leprae* make it particularly hard to study, research elucidating the virulence factors of the culturable NTM species *M. marinum* and *M. ulcerans* is advancing at a high rate. Studying the virulence mechanisms of the more pathogenic species is a good starting point for deciphering pathogenesis in opportunistically pathogenic NTM, but previously mentioned genomic studies have found that a large number of species-specific genes are present in individual mycobacterial genomes, which may leave important gaps in our knowledge of novel virulence mechanisms. These genes are prime targets for functional studies so that we may better understand their place in the survival and possibly the pathogenic potential of mycobacteria. In order to further enhance our understanding of NTM in human infection, it is imperative that the species that are increasingly implicated in human disease, such as *M. avium* and *M. abscessus*, are studied further to define the functions of genes that are currently unclear.

In addition to potential differences in virulence factors, another important difference between *M. tuberculosis* and NTM species is the presence of dynamic colony morphotypes among NTM isolates. When plated on agar media, several NTM isolates form colonies with more than one morphology; the two most common morphologies are referred to as the smooth morphotype (SM) and the rough morphotype (RM). The SM is characterized by a uniform and glossy appearance, while the RM is characterized by an irregular, dry, and corded appearance (110). The manifestation of NTM species as SM or RM distinguishes NTM from their tuberculous counterparts, the colonies of which are

predominantly rough. NTM species that exhibit both SM and RM colonies include *Mycobacterium bolletii* (111), *M. kansasii* (112), *M. abscessus* (113), *M. vaccae* (114), and *M. avium* (115). *Mycobacterium avium* colony description requires the use of additional qualifiers, as this species exhibits smooth opaque (SM-opaque), smooth transparent (SM-transparent), and RM colonies (116). Although SM and RM colony characteristics are stable over serial passages, spontaneous shifts of SM to RM and RM to SM have been reported (114, 117–119); even so, the SM is often treated in the literature as being "wild type." A genetic basis for the SM→RM transition is the lost expression of genes that promote glycopeptidolipid (GPL) synthesis and transport (111, 120). GPLs are amphiphilic molecules that localize to the outermost layer of the mycobacterial cell wall (1). GPL localization to the mycobacterial cell wall is facilitated by several genes, including *mps1*, *mbtH*, and *mmpL4b* (120). In the presence of surface GPL, a dividing mycobacterium results in daughter cells that physically dissociate. In the absence of surface GPL, a dividing mycobacterium results in daughter cells that are attached end to end. After successive divisions, these attached cells form structures that resemble cords at microscopic and macroscopic levels (114, 118). However, different species without GPL can also form SM and RM, implicating other mechanisms and membrane-associated molecules in the morphotype (114). In addition to causing RM to form corded colonies on solid media, the lack of GPL on RM causes multibacillary aggregates in liquid media (121–123). Aggregate formation can impact NTM virulence by altering the phagolysosome composition and integrity (124–126), and the RMs of *M. abscessus*, *M. avium*, and *M. kansasii* are more virulent in experimental models (112, 127, 128). Collectively, the literature cited above demonstrates that SM and RM are two physical manifestations of a single NTM species and reflect the presence (SM) or absence (RM) of surface-associated GPL in the original CFU, as a result of *mps1*, *mbtH*, and *mmpL4b* gene activity.

CONCLUSION

Nontuberculous mycobacteria are a large group of organisms that include species able to colonize human epithelia and cause disease, as well as saprophytic species that are omnipresent in soil and aquatic environments. The taxonomic and evolutionary patterns elucidated by WGS and subsequent phylogenomic analysis reviewed here have allowed us a more complete picture of the genetic similarities between different mycobacterial species. These analyses have also shed light on some interesting evolutionary characteristics of the *Mycobacterium* family, such as gene gain and loss dynamics and the immense diversity and prevalence of species-specific genes with currently unknown functions. Further study of these genes presents us with an opportunity to advance our understanding of how these bacteria colonize and may cause disease. The already well-established ability of NTM to exist in many different environments is a testament to their remarkable adaptability, and their interesting association with environmental amoebas is giving rise to very exciting ideas about the evolution of mycobacterial pathogenesis and their ability to escape macrophage killing in NTM disease. This provides a thought-provoking model of how these bacteria, and perhaps even other intracellular pathogens, may have adapted to interact with the advanced immune systems of humans and potentially cause disease. We have also summarized the current research on the unique ability of NTM to form distinct colony morphotypes that seem to differ in their virulence and pathogenicity traits, as well as their ability to form biofilms and exhibit sliding motility, which may play important roles not only in the environmental survival of NTM but also in their ability to infect human hosts. The importance of continuing research on NTM and understanding the vast number of species belonging to this group is highlighted by the increasing emergence of facultative pathogenic mycobacteria and the increasing prevalence of NTMI in immunocompetent as well as immunocompromised humans.

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